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Pyruvate dissimilation by bacterial enzyme preparations

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PYRUVATE DISSIMILATION BY BACTERIAL ENZYME PREPARATIONS

by

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INTRODUCTION

Since 1935, when the concept of CO_2 utilization in heterotrophic metabolism was first established (Wood and Werkman, 1935, 1936a), and 1938, when the relationship was demonstrated between CO_2 utilization and succinic acid formation (Wood and Werkman, 1938), considerable progress has been made towards an understanding of the fundamental role of CO_2-fixation in heterotrophic metabolism. The fixation reaction has been demonstrated with a number of heterotrophic bacteria, with yeasts, molds, and trypanosomes, and with plant and animal tissues (cf. Werkman and Wood, 1942a). The use of the isotopes, radioactive carbon, (C^{11}) and the stable form, heavy carbon (C^{13}), has confirmed the occurrence and extended our knowledge of this reaction.

In order to further elucidate the mechanism and possible physiological function of the fixation reaction, attempts have been made to utilize another tool, namely, cell-free enzyme preparations. Thus it is the purpose of this investigation to follow more accurately and in greater detail pyruvate dissimilation and heterotrophic CO_2 assimilation, using a cell-free bacterial enzyme preparation and the heavy carbon isotope. Finally, investigations will be described showing the occurrence of a compound arising from pyruvate and CO_2, and resembling oxalacetic acid, the postulated intermediate in CO_2-fixation.

HISTORICAL

For many years, investigators have tried to prepare cell-free enzyme systems from bacteria in order to obtain more information about the metabolic processes and physiological reactions going on in the interior of those cells. The review by Werkman and Wood (1940) gives an excellent account of many of those attempts. Up to a few years ago, no satisfactory method had been developed for the preparation of such enzyme systems, although a large number of different methods had been attempted. Extracellular bacterial enzymes were fairly easy to obtain from the cell-free filtrates of bacterial cultures, but generally they were present in small quantities only, and therefore had very little activity. Even after concentration of these enzymes by alcohol and acetone precipitation, this method was quite limited in scope because of the character of the enzymes involved. In another method, bacterial cells were allowed to autolyze, and attempts were then made to extract enzymes from such cells. This method, generally, was too long, stretching from days into weeks and therefore inviting contamination. Bacterial cells were also subjected to drying, shaking, alternate freezing and thawing, tryptic digestion, lysis and ultrasonic vibration, with varying success. The methods developed, generally, were too limited in scope, and applied

either to certain organisms only (as in the case of lysis), or to certain (extracellular) single enzymes and not to the majority of enzymes in the bacterial cell. With other methods, results could not be duplicated easily, and treatment was either inadequate or too drastic.

In 1938, Booth and Green developed their stainless steel mill for obtaining cell-free enzyme systems from microorganisms. The mill consists of a conical shaft rotating a set of rollers, which are in contact with a closely fitting outer race. A suspension of bacteria is then continuously circulated through the mill with the aid of a pump, and the organisms are crushed when they come between the outer guide and the revolving rollers. By the use of this mill, preparations were obtained from yeast, Sarcina lutea, Bacillus subtilis, and Escherichia coli. One ml. of E. coli juice took up 500 to 1000 µl. O_2 in one hour on hexosediphosphate in bicarbonate, and liberated approximately 1000 µl. CO_2 in one hour, anaerobically. The most active enzymes in this preparation were the dehydrogenases of malic, triosephosphoric, α-glycerophosphoric and succinic acids, as well as the hydrogenases.

This mill has been used by Gale (1939) in studying formic dehydrogenase, and by Still in his work on the alcohol dehydrogenase (1940a), on triosephosphate dehydrogenase (1940b) and pyruvate oxidation (1941). All of these preparations were obtained from E. coli.

Despite the success of the Booth-Green mill, it had the

disadvantages of being expensive and using metal surfaces for grinding. Therefore a method of grinding bacteria with powdered Pyrex glass was developed in this laboratory by Wiggert et al. (1940). The method employed equipment which was readily available, i.e., a ball mill to powder glass, and a mortar and pestle to grind the cell-glass mixture and thus disrupt the cells. With a juice prepared from Aerobacter indologenes and using the methylene blue technique, the authors demonstrated the presence of the dehydrogenases of galactose, formate, succinate, fumarate, lactate, pyruvate, xylose and dihydroxyacetone.

The disadvantage of this method was that the grinding of the bacteria, using the mortar and pestle, was done by hand. Thus, the grinding was not constant or uniform and preparations varying in activity were obtained. The method was therefore further developed by several workers in this laboratory, whereby the cell-glass paste was ground by being gently forced between two close-fitting ground-glass cones, the inner one of which was rotated. This adaptation resulted in more uniform and stronger preparations. More detailed procedures in the use of this method are given in the next section.

Using cell-free bacterial enzyme systems prepared with the aid of this glass-grinding technique, Silverman and Werkman (1941) and Gross et al. (1942, 1943) have described the conversion of pyruvate to acetylmethylcarbinol and CO_2, and Utter and Werkman have demonstrated the occurrence of the

aldolase and isomerase equilibria in bacterial metabolism (1941), the dissimilation of phosphoglyceric acid (1942a), and the effect of metal ions on the reactions of phosphopyruvate (1942b). The work with acetylmethylcarbinol was done with Aerobacter juices, the other work with E. coli enzyme systems. In the present report, a cell-free enzyme preparation, obtained from E. coli by the same method, was used to study the anaerobic dissimilation of pyruvate, to determine the components of the enzyme system concerned, and to compare the enzymes functioning in the anaerobic dissimilation of pyruvate with those responsible for its aerobic breakdown. During the anaerobic dissimilation of pyruvate, the utilization of carbon dioxide was demonstrated with this preparation.

Pyruvate as a Metabolic Intermediate

> ". . . . There is no intermediary substance produced during the metabolism of foodstuff that possesses the reactivity of pyruvate, and is able to take part in such a variety of reactions. Pyruvate is thus the hub toward which converge carbohydrate, fats and proteins in their catabolic and anabolic reactions."
> (Barron, 1943)

In the oxidation of fat, the molecule is probably oxidized to glycerol and fatty acids, and these two components then undergo oxidation. There is reason to believe that the intermediate steps in the oxidation of glycerol resemble those in the oxidation of carbohydrates. In all probability, the glycerol, through such stages perhaps as glyceraldehyde, pyruvic

acid or dihydroxyacetone is finally broken down to CO_2 and H_2O (Harrow, 1943). There is evidence, also, that fatty acids, in metabolism, can be oxidized to a four-carbon stage, acetoacetic acid, and there is some further evidence that this can be converted into acetic acid in the tissues (Harrow, 1943). It is further suggested (cf. Krebs, 1943) that butyric acid arising from fatty acids undergoes gamma oxidation and is converted into succinic acid. The relationship of succinate to pyruvate through fumarate, malate and the decarboxylation of oxalacetate is already fairly well established. Liver forms acetoacetic acid from pyruvate (Barron et al., 1941), and it is suggested that the synthesis of carbohydrate from fat probably occurs through the link between acetoacetic acid and pyruvic acid.

The conversion of carbohydrate into fat has been known to occur for a long time. Here, our information as to the mechanisms involved is little more than guess work. It has been postulated that carbohydrate is converted to pyruvic acid and the pyruvic acid then decarboxylated to acetaldehyde. From there, by a series of condensations, either between one molecule of pyruvate with one molecule of acetaldehyde or between two molecules of acetaldehyde, eventually, 16- and 18- carbon fatty acids would be reached. As some evidence that pyruvate might be a factor in the conversion of carbohydrate into fat, thiamine and two other factors, riboflavin and pantothenic acid are apparently required for this reaction (Harrow, 1943). Of approximately fourteen reactions which pyruvate is known to

undergo, diphosphothiamine has been found necessary for eleven of those reactions (Barron, 1943).

The inter-relationships of proteins and pyruvate are more obvious. For example, a number of amino acids yield breakdown products during metabolism, which products also arise from carbohydrate. For example, glutamic acid, on transamination, yields ketoglutaric acid, which is decarboxylated to succinic acid, and then can easily be converted to pyruvate. Aspartic acid on transamination yields oxaloacetate, which, on decarboxylation also yields pyruvate. Alanine, during transamination, can be directly converted to pyruvate. Altogether, at least eight or nine amino acids yield pyruvate on oxidation (Krebs, 1943). In the conversion of carbohydrate into protein, pyruvate could again logically serve as a key intermediate, through carboxylation, the Krebs tricarboxylic acid cycle, and transamination.

In carbohydrate metabolism, during the breakdown of glucose to pyruvate, there are eight separate steps, all of them reversible except the last: the conversion of phosphopyruvate to pyruvate. All these reactions are anaerobic. Therefore, pyruvate is the end product of fermentation in normal, complete, carbohydrate metabolism. From this point, the breakdown of pyruvate may proceed under anaerobic or aerobic conditions, and may take a number of different paths in different tissues and under different conditions.

In conversion of fats and proteins into carbohydrate,

phosphopyruvate is probably the more logical intermediate, although pyruvate itself can be converted into glycogen (Buchanan et al., 1942).

Reactions of pyruvate

According to its important position in cellular metabolism, pyruvate should be, and is, a very reactive compound. Its many reactions have been found in animal tissues, yeast, bacteria, fungi, and plants. Pyruvate undergoes the following reactions:

1. Oxidation in muscle. For every molecule of pyruvate utilized, 2-1/2 molecules of O_2 are taken up and three molecules of CO_2 are formed:

$$CH_3.CO.COOH + 5O = 3CO_2 + 2H_2O$$

2. Oxidative decarboxylation in animal tissues and bacteria:

$$CH_3.CO.COOH + O = CH_3.COOH + CO_2$$

3. Transamination in animal tissues:

$CH_2.COOH$ CH_2 $CH(NH_2).COOH$	+	$CH_3.CO.COOH$	$\rightleftharpoons$	$CH_2.COOH$ CH_2 $CO.COOH$	+	$CH_3.CH(NH_2).COOH$	
Glutamic acid		Pyruvic acid		α-Keto-glutaric acid		d-Alanine	

4. Condensation to acetoacetate in liver.

5. Decarboxylation in yeast:

$$CH_3.CO.COOH = CH_3.CHO + CO_2$$

6. Reduction in muscle and bacteria:

$$CH_3.CO.COOH + 2H \rightleftharpoons CH_3.CHOH.COOH$$

7. Dismutation in muscle and bacteria:

$$2CH_3.CO.COOH + H_2O = CH_3.CHOH.COOH + CH_3.COOH + CO_2$$

Among the bacteria, this dismutation is carried out by Staphylococcus aureus, Staph. albus, Streptococcus fecalis, Neisseria gonorrhoea, Lactobacillus delbruckii, and E. coli at low pH.

8. Hydroclastic reaction in bacteria:

$$CH_3.CO.COOH + H_2O = CH_3.COOH + HCOOH$$

Occurs in E. coli, Strep. hemolyticus, Aerobacter aerogenes and Proteus vulgaris.

9. Oxidation with water by bacteria (Clostridium)

$$CH_3.CO.COOH + H_2O = CH_3.COOH + CO_2 + H_2.$$

In this case, CO_2 and H_2 are produced, instead of formic acid. However, it is unlikely that the CO_2 and H_2 arise from formic acid because formic acid is not attacked by these bacteria.

10. Condensation to acetylmethylcarbinol (Aerobacter)

$$2CH_3.CO.COOH \rightleftharpoons CH_3.CHOH.CO.CH_3 + 2CO_2$$

11. CO_2-fixation or carboxylation:

$$CO_2 + CH_3.CO.COOH \rightleftharpoons HOOC.CH_2.CO.COOH$$

CO_2-fixation gives a series of products and thus makes possible a large number of reactions. At this point it might be well to present a short summary of the events and results that led to the acceptance of the active participation of CO_2 in heterotrophic metabolism.

CO_2 utilization

It is generally known that autotrophs can assimilate CO_2 and utilize it as their sole source of carbon by the use of photosynthetic and chemosynthetic mechanisms. By contrast, up to few years ago, it was believed that animals and other heterotrophic non-photosynthetic organisms could not utilize CO_2, but used organic carbon compounds to satisfy their carbon needs.

More recently, however, it has been demonstrated that heterotrophic bacteria can utilize CO_2 to form carbon-to-carbon linkages, but their carbon needs cannot be met by this compound alone. Heterotrophic CO_2-fixation, since its discovery by Wood and Werkman in 1935, has since been extended to yeasts, molds, and trypanosomes, and has also been shown to take place in plant and animal tissues. (For a complete treatment of this subject, see the review by Werkman and Wood, 1942a.) Recent reviews on metabolism, including CO_2 utilization are those by Werkman and Wood (1942b), Barron (1943), and Krebs (1943).

The heterotrophic utilization of CO_2 was reported while a study was being made of the dissimilation of glycerol by several species of Propionibacterium with $CaCO_3$ as a buffering agent. The total carbon dioxide liberated during the fermentation, plus that remaining as carbonate, was less than the CO_2 originally added as carbonate. Oxidation-reduction indexes and carbon balances supported this view.

The first important step in elucidating the mechanism of this reaction was evidence that CO_2 utilization took place by addition of a three-carbon compound (C_3) to a one-carbon compound (C_1), to form succinic acid, a four-carbon compound (Wood and Werkman, 1938). Since the succinic acid was formed from glycerol in equimolar quantities to the CO_2 fixed, the synthesis by C_3 and C_1 addition seemed probable. Furthermore, pyruvate was proposed as the C_3 compound of the fixation reaction, since it could be isolated from the fermentation liquor by addition of bisulfite (Wood and Werkman, 1937). It was further proposed that the resulting oxalacetate could be reduced stepwise to succinic acid according to the following reactions:

$$CO_2 + CH_3.CO.COOH = COOH.CH_2.CO.COOH$$

$$COOH.CH_2.CO.COOH + 4H = COOH.CH_2.CH_2.COOH + H_2O$$

(Wood et al., 1941).

Another indication as to the mechanism of the reaction was presented by Elsden (1938) who showed that succinic acid formation by E. coli was dependent on the concentration of CO_2 in the medium. This was also the first extension of CO_2-fixation to another heterotrophic organism. Thus, from the following facts more direct evidence was obtained for the utilization of CO_2 in the formation of succinic acid by heterotrophic bacteria: (1) that there was a direct equimolar relationship between the CO_2 utilized and the succinic acid formed, and (2) succinic acid formation depended on the

concentration of CO_2 in the medium, and (3) there was little or no succinate formed when the glycerol fermentation was carried out in phosphate buffer, in the absence of CO_2 (Wood and Werkman, 1938).

In 1940 (a), Wood and Werkman demonstrated the formation of succinate by propionic acid bacteria, from a number of substrates. Further proof that pyruvate was (or could be converted into) the three-carbon compound which actually partook in the fixation reaction was presented in several other papers. In 1941 (a), Wood et al. demonstrated the fixation of CO_2 by coliform bacteria, during the fermentation of galactose, citrate, and pyruvate. In subsequent studies on CO_2-fixation pyruvate was used as substrate by several different investigators.

Confirmation of the occurrence of the fixation reaction during the dissimilation of glycerol by the propionic acid bacteria was presented by Phelps et al. (1939), by Carson and Ruben (1940) using radioactive carbon (C^{11}), and by Wood et al. (1940) employing the stable isotope (C^{13}).

The availability of carbon isotopes at this time greatly facilitated further study of the mechanism of this reaction. With the aid of the isotopes, in the form of $NaHC^{13}O_3$ added to the medium, an excess of heavy carbon was found to be present in the succinic acid formed during CO_2-fixation. By degrading the succinic acid molecule, the excess C^{13} was found to be present exclusively in the carboxyl groups of the succinic acid

(Wood et al., 1941b). Since succinic acid is a symmetrical molecule, differentiation of the C^{13} content of each carboxyl group could not be made, but calculations made on the basis of the C^{13} available and the actual amount of C^{13} fixed indicated that all of the fixed carbon was located in one carboxyl group of succinic acid. According to the following postulated mechanism, the labelled carbon (C^{11} or C^{13}) should be in only one carboxyl group of the final succinic acid formed:

$$\dot{C}O_2 + CH_3.CO.COOH \longrightarrow \dot{C}OOH.CH_2.CO.COOH \xrightarrow{+2H} \dot{C}OOH.CH_2.CHOH.COOH.$$

$$\dot{C}OOH.CH_2.CHOH.COOH \xrightarrow{-H_2O} \dot{C}OOH.CH{:}CH.COOH \xrightarrow{+2H} \dot{C}OOH.CH_2.CH_2.COOH.$$

Further proof of this postulated series of reactions came when Nishina, Endo and Nakayama (1941), using radioactive carbon, demonatrated the formation of malic and fumaric acids from pyruvate by E. coli.

By the use of the heavy carbon isotope, it was also determined that the propionic acid formed during the fermentation of glycerol by the propionic acid bacteria also contained heavy carbon (Wood et al., 1940). In attempts to elucidate this mechanism, further work seemed to point to the probability that propionic acid may be formed in this fermentation by the unexpected mechanism of union of a three-carbon compound with CO_2, followed by reduction to succinate and decarboxylation to propionic acid (Wood et al., 1941a). Slade et al. (1942) showed that CO_2 was assimilated by a wide variety of heterotrophic bacteria with formation of a carbon-to-carbon

linkage. With C^{13} as a tracer, fixed carbon was also found to be present in the carboxyl group of lactic acid formed by several species of bacteria. Several possible mechanisms were advanced to explain this fact, including one somewhat similar to that proposed for the formation of propionic acid. In experiments with the homolactic acid bacteria, no fixation occurred. Furthermore, some genera formed acetic acid containing heavy carbon in the carboxyl group, and the authors postulated its occurrence through a cleavage of a previously formed C_4 dicarboxylic acid.

Extension of the concept of heterotrophic CO_2 utilization to animal tissues came about quite logically, and in the last few years, much work has been done in this field, by several different groups of investigators. In 1938, when it was proposed that pyruvic acid was the possible three-carbon intermediate compound undergoing fixation, Wood and Werkman also suggested that this fixation might take part in Krebs' citric acid cycle.

The Krebs cycle was originally proposed by Krebs and Johnson (1937), and modified by Wood et al. (1941c, 1942) and by Evans and Slotin (1941). It was proposed as a mechanism to account for the complete oxidation of a molecule of pyruvate to CO_2 and H_2O by pigeon breast muscle. In that scheme, one molecule of oxalacetic acid unites with one molecule of pyruvic acid to form a seven-carbon compound, which then passes through a series of oxidative decarboxylations until the end product,

oxalacetic acid, is reached. Thus, the carbon and hydrogen of the pyruvic acid molecule are oxidized to CO_2 and water, and the oxalacetate regenerated again.

The scheme, as presented by Krebs, also included the mechanism for hydrogen transport proposed by Szent Györgyi and co-workers (1937). These investigators suggested that catalytic quantities of the four-carbon dicarboxylic acids acted as hydrogen transporters in the following manner: oxalacetic acid accepts hydrogen (donated by some other substrate during metabolism) and thus is converted to malic acid; the malic acid, through successive steps, passed through furmaric and succinic acids, and from the latter compound the hydrogen is passed on to cytochrome oxidase and finally to oxygen, to form water. Thus, in the oxidation of triosephosphate, oxalacetic acid accepts hydrogen and is converted to malate. According to Krebs (1943) two other reactions can donate hydrogen to oxalacetate: the conversion of the intermediate seven-carbon compound (pyruvo-fumaric acid) to cis-aconitic acid, and the oxidation of isocitric through oxalosuccinic to α-ketoglutaric acid.

In order for carbohydrate to be oxidized by this scheme, i.e., pyruvate to be converted to CO_2 and H_2O, the presence of oxalacetic acid is necessary. The actual amount of oxalacetate necessary would be catalytic, and could arise from either of two types of sources or from both. One source might be glutaric and aspartic acids, and citrate, succinate, and malate

from protein or carbohydrate metabolism. The other possible source of oxalacetate might be from the carboxylation of pyruvate, i.e., the Wood-Werkman reaction.

The Krebs cycle has been demonstrated principally in pigeon liver and muscle. However, further accumulation of data also seems to indicate the occurrence of this cycle in sheep heart, testis, brain, and guinea pig kidney. Part of this cycle, i.e., the conversion of citrate to α-ketoglutaric acid has also been observed in plant tissue (cucumber seeds, Krebs, 1943). The reactions in pigeon liver and muscle were observed to be quite similar, but not identical. For example, the addition of malonic acid to pigeon breast muscle stops the dissimilation of pyruvate by that tissue. Malonic acid inhibits the succinic dehydrogenase which converts succinate to fumarate and therefore prevents the regeneration of oxalacetate necessary for the Krebs cycle. Addition of fumaric or malic acid relieves the inhibition caused by the malonate, because these acids are easily converted to oxalacetate. This conversion allows the dissimilation of pyruvate to proceed, but only proportionally to the amount of C_4 acid added, because no oxalacetate is regenerated, due to the presence of the malonate. As mentioned, addition of malonate stops pyruvate dissimilation with muscle tissue, but not with liver. Therefore it seemed evident that there must be another method of synthesizing oxalacetate, in liver tissue.

Further indirect evidence for the occurrence of the carboxylation reaction in animal tissue came when Evans (1940)

showed that pigeon liver carried on the Krebs cycle with pyruvate as the only substrate. As both pyruvate and oxalacetate are needed, the oxalacetate obviously had to be formed from the pyruvate. Krebs and Eggleston (1940, 1941) showed that carbon dioxide stimulated succinic acid formation from pyruvate by pigeon liver. The authors were of the opinion that, since pyruvate oxidation by the pigeon liver depended upon the concentration of CO_2 and bicarbonate, the CO_2 was used in the synthesis of oxalacetate, from pyruvate, both then combining to undergo the various changes of the Krebs cycle. More direct evidence of this reaction was presented by Evans and Slotin (1940) who showed with the aid of radioactive carbon that CO_2 was fixed in the α-ketoglutaric acid formed from pyruvic acid by pigeon liver.

Wood et al. (1941), with the aid of tracer technique, and then Evans and Slotin (1941) showed the position of the fixed carbon dioxide in the α-ketoglutaric acid to be only in the carboxyl group adjacent to the carbonyl group. Since citric acid is a symmetrical molecule, it had been postulated that the fixed carbon would be found in both carboxyls of the α-ketoglutarate. Thus, the results necessitated a modification of the metabolic scheme, in which citric acid is no longer a principal in the cycle but is relegated to a side path similar to the formation of lactate from pyruvate.

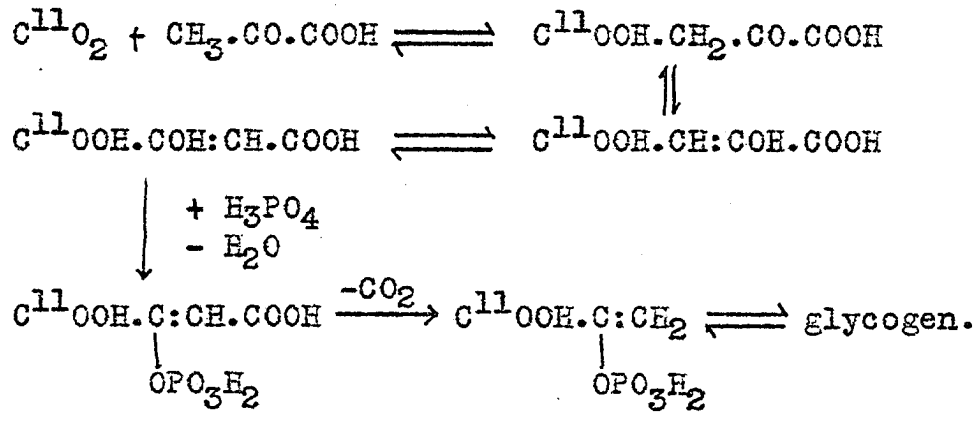
Wood et al. (1942) also conclusively showed that there are at least two mechanisms for the formation of succinate in

liver, one aerobic and the other anaerobic. The Krebs cycle adequately accounts for the aerobic formation of succinate from pyruvate. In the presence of malonate, the succinate formed from the decarboxylation of α-ketoglutarate contained no heavy carbon. The carboxyl containing the heavy carbon is lost, and consequently the C^{13} content of the succinate was normal. However, due to CO_2 fixation, the malate and fumarate formed, did contain an excess of heavy carbon, but were prevented from being converted into succinate by the malonate present.

Thus, from the evidence cited, it appears that carboxylation does occur in pigeon liver, anaerobically, and pyruvate dissimilation therefore proceeds without the addition of a four-carbon dicarboxylic acid. On the other hand, carboxylation does not occur in muscle, at least not at a rate comparable with that in liver, because muscle, unlike liver, does not synthesize significant quantities of ∝-ketoglutaric acid from pyruvate in the absence of added oxalacetate or malate.

Further observations on carbon dioxide assimilation by animal tissues have been presented in a series of papers from Hasting's laboratory. Solomon et al. (1941) have shown that when lactate is fed to fasted rats, and radioactive bicarbonate is injected intraperitoneally, the liver glycogen formed contains radioactive carbon. As a probable mechanism, the authors postulate the carboxylation of pyruvate, phosphorylation of the four-carbon dicarboxylic acid, decarboxylation to phosphopyruvate and subsequent synthesis to glycogen through

reverse glycolysis:



Continuing this work, Vennesland et al. (1942) further demonstrated that on feeding glucose to fasted rats, with radioactive bicarbonate present, the synthesized glycogen contained some of the radioactive carbon. In another communication (a preliminary report), Buchanan et al. (1942) demonstrated glycogen synthesis from pyruvate with rabbit liver slices, again using the tracer technique.

The carboxylation reaction has also been extended (by analogy) to pigeon liver, brain, and guinea pig kidney cortex, which appear to slowly synthesize, glutamine from ammonium pyruvate (Örström et al., 1939; Weil-Malherbe, 1936; Krebs, 1943).

Following these indications of the wide biological occurrence of CO_2-fixation, the natural consequence of the investigations which followed was an attempt to elucidate the mechanism by which carbon dioxide enters metabolic processes.

Mechanism of the Carboxylation Reaction

Following the hypothesis that the fixation reaction took place by a three-carbon and one-carbon addition, pyruvic acid was proposed as the three-carbon constituent, which after condensation with carbon dioxide, yielded oxalacetic acid.

Since diphosphothiamine had been found necessary for many reactions which pyruvate undergoes, the evidence which Smyth (1940) and Krebs and Eggleston (1940) presented as to the necessity of diphosphothiamine for the fixation reaction seemed quite logical. Smyth, working with staphylococci found that oxalacetate replaced thiamin in the dismutation of pyruvate to lactate, acetate, and CO_2. Therefore, it was concluded that thiamin was required for the synthesis of oxalacetate, which could then act as a hydrogen acceptor in the dismutation of pyruvate.

Krebs and Eggleston, investigating the dissimilation of pyruvate by pigeon liver, found that CO_2 was stimulatory and observed that thiamin also had the same effect, i.e., stimulated dissimilation of pyruvate by liver from an avitaminous pigeon. They concluded that pyruvate and CO_2 united to form oxalacetate, and that the vitamin was necessary for this synthesis. The authors gave further weight to these observations when it was found that the vitamin did not stimulate pyruvate dissimilation by pigeon breast muscle obtained from the same avitaminotic bird. This was considered important in view of the

postulated occurrence of the fixation reaction in pigeon liver, but not in breast muscle.

These findings were not substantiated by Krampitz and Werkman (1941). With an acetone preparation obtained from Micrococcus lysodeikticus which catalyzed the reaction $COOH.CH_2.CO.COOH \longrightarrow CO_2 + CH_3.CO.COOH$, they found that magnesium ions were required for the reaction, whereas cocarboxylase and thiamin had no effect. However, both Mg^{++} and diphosphothiamine were essential for pyruvate oxidation. With oxalacetate as the substrate, there was a sufficient supply of pyruvate, owing to the spontaneous decarboxylation of oxalacetate, and yet, in the presence of the C_4 acid, diphosphothiamine was still necessary for pyruvate oxidation.

There may be several explanations for the discrepancies observed in the results of these three groups of investigators. Smyth did not present conclusive proof of the necessity of the vitamin for the formation of oxalacetate, for we know of one instance at least where both diphosphothiamine and a C_4-dicarboxylic acid (besides phosphate, Mg^{++} and adeninedinucleotide) are necessary for aerobic pyruvate dissimilation (Banga et al., 1939). (It may be added at this point that present evidence strongly indicates that anaerobic and aerobic pyruvate dissimilation are quite similar as to components of the enzymes involved.) Therefore the possibility exists that the vitamin-deficient staphylococci used by Smyth had a multiple deficiency, and thus stimulations were observed on addition

of both diphosphothiamine and oxalacetate. Recently, Krebs and Eggleston (1941) reported that the addition of a C_4-dicarboxylic acid stimulated the fermentation of glycerol by propionic acid bacteria. As the fixation reaction is known to occur with these bacteria oxalacetate should be formed. Yet there was stimulation on addition of this compound. This serves to illustrate that stimulation may result, not because of a weak fixation reaction, but for some other reason.

The evidence presented by Krebs and Eggleston as to the necessity of cocarboxylase for the fixation reaction is entirely indirect since it was not shown to be directly associated with an uptake of CO_2. Furthermore, no proof was presented that the breast muscle was deficient in thiamine. It is known also that all organs of an avitaminotic animal are not equally deficient in the vitamin (Ochoa and Peters, 1938). Thus it is still possible that, whereas the liver was deficient in the vitamin, the breast muscle might not have been. This might explain why thiamin stimulated pyruvate dissimilation in liver and not in muscle. It has also been pointed out that minced muscle is unable to phosphorylate thiamine rapidly (Ochoa, 1939). It has also been pointed out by Krampitz and Werkman that the acetone preparation of M. lysodeikticus utilizes cocarboxylase, but not thiamine, which indicates that the mechanism for phosphorylating the vitamin is destroyed by acetone treatment.

Thus, there seems to be no direct evidence that

cocarboxylase is a component of the enzymes concerned with CO_2-fixation, for the simple reason that in all instances the evidence has been indirect and the fixation reaction is but one of the reactions taking place in the cells or tissues studied. Furthermore, in no instance was carboxylation of pyruvate actually shown to take place, nor an uptake of CO_2 demonstrated.

The increasing biological importance of heterotrophic CO_2 assimilation has led to further speculation as to possible co-enzymes of this reaction. Thus, Burk and Winzler (1943), reflecting on the fundamental function of biotin in living matter, found some evidence for postulating a biotin vitamer (i.e., a substance which acts to overcome a biotin deficiency) as a possible co-enzyme in this reaction. Evidence was the existence of a urea ring that may be opened or closed either chemically or biologically by yeast with respective loss and gain of CO_2, and the fact that the CO_2 pressure requirements for growth in the presence of diaminocarboxylic acid, derived from biotin, is much greater than in the presence of biotin. Maximum DAC activity at limiting DAC concentrations requires a CO_2 pressure greater than that in air. All this suggested the interesting possibility that biotin and its vitamers could act, possibly as a co-enzyme of CO_2 transfer, either in CO_2-utilization or CO_2-production, similar to the functions of co-enzymes in hydrogen transfer, and adenylic acid in phosphate transfer.

Barron (1943) presented some evidence that -SH groups might function as a co-enzyme in CO_2-utilization. The activating protein of the following enzyme systems were found to contain -SH groups essential for enzyme activity: pyruvate oxidation, pyruvate condensation to carbohydrate, acetoacetate and α-ketoglutarate, malic oxidase, α-ketoglutaric oxidase, d-amino acid oxidase, and succinoxidase. In view of the evidence already presented for the formation of glycogen containing radioactive carbon, from pyruvate and $NaHC^{11}O_3$ (Buchanan et al., 1943), and furthermore, that glutathione (-SH groups) has been found necessary for pyruvate condensation to carbohydrate, it was suggested that perhaps this group is necessary for the Wood-Werkman reaction.

The only definite evidence as to possible components of the fixation reaction has been obtained by Krampitz et al. (1943). These authors did determine that magnesium ions are necessary for carboxylation. After obtaining an acetone preparation from Micrococcus lysodeikticus which catalyzed the decarboxylation of oxalacetate to pyruvate and CO_2, their further attempts to demonstrate the carboxylation of pyruvate were not successful. However, it was recognized that the possibility of carboxylation was not excluded, since the equilibrium of the reaction might be far to the side of decarboxylation and therefore the quantities of oxalacetate formed would be too small for detection. Their results showed that in the presence of the enzyme a reversible exchange took place between

the $C^{13}O_2$ of the medium and the carboxyl carbon adjacent to the methylene carbon of oxalacetate and that Mg^{++} was necessary for this reaction.

They also found that starting with oxalacetate obtained from the oxidation of fumarate, a greater exchange occurred between the labile carboxyl group of oxalacetate and the $C^{13}O_2$ of the medium, indicating that this "physiological" oxalacetate was different from the ordinary chemical form. No exchange occurred in the other carboxyl group of oxalacetic acid and also little or no exchange took place in the absence of the enzyme. Thus, these results constitute the first direct evidence that oxalacetic acid is a component of the fixation reaction and that the reaction $CO_2 + CH_3.CO.COOH \rightleftharpoons HOOC.CH_2.CO.COOH$ is reversible.

The fact that no C^{13} was found in the other carboxyl group of oxalacetic acid was evidence against the occurrence of a dynamic equilibrium involving a shift of the hydroxyl group of enol-oxalacetate, as proposed by Meyerhoff (1941). No exchange occurred during the oxidative decarboxylation of pyruvate or α-ketoglutarate, nor did pyruvate derived from lactate show exchange. Evans (1942), using yeast carboxylase, found no exchange of $C^{11}O_2$ with pyruvate, and Ruben and Kamen (1940) also considered this same reaction irreversible. However, Carson et al. have recently stated that a very small amount of $C^{11}O_2$ is utilized by carboxylase preparations in the presence of acetaldehyde and CO_2. Therefore, in view of

the results on enzymatic exchange obtained by these investigators, the results of Krampitz et al. (1943) are further evidence of the validity of the fixation reaction as proposed by Wood and Werkman.

Oxalacetic acid or probably a phosphorylated form is the key intermediate in the fixation reaction. Up to the present time, however, no one has demonstrated the actual formation of oxalacetate from pyruvate and CO_2. The results of Krampitz et al. (1943) in demonstrating a slight carboxylation during the decarboxylation of oxalacetate is the first direct evidence that oxalacetic acid may be a component of the fixation reaction. Also the fact that physiological oxalacetate, resulting from fumarate oxidation, was more stable and gave higher exchange values with C^{13} leads to the belief that the oxalacetate concerned in the fixation reaction is different from the chemically synthesized compound.

In the opinion of some investigators (Krebs, 1943), the conclusion that pyruvate is converted to oxalacetate during the fixation reaction is considered inescapable, and is based on the following facts, obtained from work done with pigeon liver: the substances arising from pyruvate in pigeon liver are the same as those formed on addition of oxalacetate; since both pyruvate and oxalacetate form fumarate, malate, succinate, α-ketoglutarate, and citrate in the same definite proportions, the simplest explanation involves the formation of oxalacetate by carboxylation of pyruvate. This explanation

is supported by observations that the rate of pyruvate removal and α-ketoglutarate formation are dependent on the concentration of bicarbonate and CO_2. The instability of oxalacetate and its rapid dissililation lead Krebs to the conclusion that "the prospects of a successful isolation of the oxalacetate formed by the /fixation/ reaction are very slight, if not nil."

Block and Barron (Barron, 1943) attempted to orient the metabolism of pyruvate toward the formation of oxalacetate by increasing the CO_2 tension and decreasing the temperature to 20°C, to avoid oxalacetate decomposition. Although there was vigorous pyruvate utilization in liver slices of rat and pigeon, no oxalacetate was detected.

In the present report, after at least two mechanisms for CO_2-fixation and the formation of succinic acid were demonstrated, attempts were made to prevent the reduction of any possible oxalacetate formed during the fixation reaction, and thereby cause it to accumulate in order to demonstrate its occurrence. With the citrate-aniline method, and a colorimetric method, definite tests were obtained for the formation, from pyruvate and CO_2, of a compound resembling oxalacetate. The amounts of oxalacetate (or its derivative) detected, although quite small, were found to vary with the concentration of enzyme and pyruvate, and the presence of carbon dioxide was found necessary for its formation.

METHODS

Growth Media

In the work preliminary to obtaining cell-free juices active in the dissimilation of glucose, Escherichis coli (26) was grown in 10-liter quantities of the following medium: 1% glucose, 0.4% peptone, 0.8% K_2HPO_4, and 10% tap water. The enzyme preparations which were active on pyruvate were obtained from E. coli grown in 10 liters of a medium containing 0.3% beef extract, 0.3% peptone, 0.3% yeast extract, 0.2% NaCl, 10% tap water, plus distilled water to volume. The 24-hour growth of cells on a nutrient agar slant was washed off with sterile water, and this suspension used as an inoculum. The inoculated medium was continuously and strongly aerated for 24 to 36 hours at 30° C, and a small amount of octadecyl alcohol was added to prevent excessive foaming of the medium caused by strong aeration. There was very little growth in this medium in the absence of aeration. Furthermore, strong aeration was desired (Yudkin, 1932) so that the resulting cells would not contain the enzyme hydrogenlyase; i.e., formic acid would not be broken down to CO_2 and H_2.

The cells were harvested by centrifugation in a Sharples supercentrifuge, run at about 35,000 revolutions per minute. The yield was usually between 10 and 25 grams of cells per

10 liters of medium. Attempts to increase the yield by varying the concentrations of the constituents failed. E. coli, when grown in a sugar medium, produces little or no succinic acid, and for this reason, sugar was not used. The yield is not appreciably increased when the cells are allowed to grow for more than 30 hours. However, it was found that the nutrients in the medium were not exhausted after growth had taken place, for on re-sterilization and re-inoculation, a second crop of cells of approximately the same weight as the first, was harvested.

The Enzyme Preparation

The cells (wet weight, as obtained from the Sharples Centrifuge) were mixed with powdered glass, obtained by grinding small, clean pieces of Pyrex glass in a ball mill containing steel balls. The powdered glass was then passed through a set of sieves, which prevented glass particles larger than 1/2 mm. in diameter from passing. The average size of the glass particles was about two microns. The original method of grinding bacteria with powdered glass, using a mortar and pestle, was developed by Wiggert et al. (1940) and since then has been further developed in this laboratory, as described below. To every gram of wet cells, two grams of ground glass were added, and the resulting cell-glass paste mixed until it resembled a rather firm batter.

The cell-glass paste was then ground by being gently

forced between two close-fitting ground-glass cones. The inner cone was attached to a strong motor and rotated steadily at approximately 150 revolutions per minute, while the outer cone was held in a fixed position. A short piece of thick rubber tubing served as a connection between the inner cone and the rotating shaft of the motor, and gave the system the flexibility necessary to achieve intimate contact between the two ground-glass surfaces, with much less danger of breakage. The grinding surfaces were cooled by filling the inner cone with crushed ice.

The ground material was collected in a beaker surrounded by crushed ice, and extracted by thoroughly mixing it with 0.2 M. phosphate buffer (pH 6.82-6.88) or, in a few experiments, with distilled water. One and five-tenths milliliters of extract buffer or water was added for every gram of wet bacterial cells originally employed.

The glass and larger cell particles were thrown down by centrifugation on an International or angle centrifuge at approximately 3,500 revolutions per minute for five minutes. The supernatant liquid was further clarified in an air-driven Beam's ultracentrifuge (Beams, 1930) at 60 to 64 pounds air pressure, rotating at approximately 75,000 to 100,000 revolutions per minute. The cell fragments were deposited on the wall of the rotating cup, and the clear liquid was removed from the center of the cup by means of a capillary pipette. Centrifugation for ten minutes generally gave a clear juice.

The final enzyme preparation generally was a clear, yellow-brown liquid, slightly viscous and opalescent.

Preparations obtained from cells grown on the solid agar medium of Krebs (1937) had only a weak activity and did not have as much color or viscosity as the juices obtained from cells grown in liquid culture. The latter organisms seemed less resistant to grinding and consistently yielded stronger enzyme preparations.

Manometric Methods

The conventional Barcroft-Warburg apparatus was used in most of the experiments. The following are some examples of the use of the Warburg apparatus: (1) small-scale fermentations, in which only pyruvate dissimilation and CO_2 evolution were to be determined; (2) quantitative measurement of CO_2-fixation, O_2 consumption and H_2 uptake; (3) determination of the activity of enzyme preparations and the optimum conditions for such activity; (4) determination of the presence of different enzyme systems in the preparation and components of the systems (after dialysis). In general, 0.8 ml. of the enzyme preparation, plus buffer, was placed in the main chamber of each flask. The substrate was placed in one sidearm, and any other constituent of the reaction mixture was placed in the other sidearm of the vessel. Water was added to bring the total volume to 2.0 or 2.3 ml. When it was necessary to absorb

evolved CO_2, 0.3 ml. of 20% NaOH was placed in the alkali chamber of the Warburg flask, plus folded filter paper to increase the absorbing surface. The air above the liquid in each flask was replaced with the required gaseous atmosphere. After temperature equilibration, readings were taken and the substrate tipped into the main chamber containing the enzyme. The enzyme preparation had very little endogenous activity, but to check analytical procedures, controls were always run. All experiments were carried out at 30.4° C.

Large Scale Fermentations

When complete fermentation analyses were to be performed, the experiments were carried out in one of two ways: (1) in a 125-ml. two-sidearm flask containing 30 ml. of reaction mixture attached to a Warburg manometer to follow the course of the enzyme action, or (2) in a stationary 300-ml. Erlenmeyer flask connected to a condenser and then to a bead tower containing 15 ml. of 1.5 N CO_2-free NaOH. The air above the fermentation was displaced with CO_2-free H_2, and the whole system put under a slight negative pressure. In this way, all the CO_2 liberated during the course of the experiment was collected in the bead tower containing the NaOH. The Erlenmeyer flask contained approximately 70 ml. of reaction mixture, and the experiments were carried out at 30° C.

Analysis of Fermentation Liquors

Pyruvic acid

Residual pyruvate from large or small scale fermentations was determined colorimetrically by the salicylaldehyde method of Straub (1936). The fermentation mixture (or the contents of a Warburg flask) was acidified with sulfuric or trichloracetic acid and diluted so that one milliliter contained not more than 0.5 mg. of pyruvic acid. The proteins were filtered off, and to one milliliter of the unknown, 1.0 ml. of KOH solution (100 gm. KOH + 60 gm. H_2O) and 0.5 ml. of salicylaldehyde solution (2% salicylaldehyde, by volume, in 100 ml. of 95% alcohol) were added; the contents were mixed thoroughly and kept in a water-bath at 37° C for 10 minutes. The contents of the test tube were then cooled to room temperature, centrifuged off from any K_2SO_4, and read within an hour in a Klett-Summerson photoelectric colorimeter with the 470 mμ. filter. Reagent controls and known pyruvate samples were run with each test. Acetaldehyde and acetone will interfere with this test.

The pyruvic acid used was freshly distilled under vacuum. The second fraction was collected and kept in the crystalline form under refrigeration. Solutions of sodium pyruvate were kept frozen when not in use. Pyruvate kept in this way for several months was 80-90% utilized by the enzyme system. The yield of sodium pyruvate when prepared according to Peters (1938) was low but was completely utilized; whereas with the

method of Robertson (1942) sodium pyruvate was easily prepared, but the enzyme system utilized this sodium pyruvate only to a very limited extent.

A qualitative colorimetric method was used for the detection of pyruvate in the volatile acid distillate during fermentation analyses. Pyruvic acid is volatile with steam to some extent, and two or three steam distillations of the volatile acids were sometimes necessary to remove it. To determine whether pyruvic acid has effectively been removed, two milliliters of the volatile acid fraction were saturated with ammonium sulfate. Four drops of a 2% sodium nitroprusside solution were added, plus 1.0 ml. of conc. NH_4OH. A blue color indicated the presence of pyruvic acid (Simon and Piaux, 1924). Other keto acids, such as α-ketoglutaric and oxalacetic acid, will give different color reactions.

Volatile acids

For the determination of volatile acids, the fermentation was neutralized, evaporated to ten milliliters, acidified to congo red, and fifteen volumes steam distilled. The distillate was refluxed, an aliquot removed and titrated for total volatile acids with 0.05 N NaOH.

Formic acid was determined qualitatively by neutralizing two milliliters of the volatile acid distillate with NH_4OH. Then, a few drops of $AgNO_3$ were added, and the solution heated in a steam bath for five minutes. The formation of a silver

mirror was a qualitative test for the presence of formic acid. Quantitatively, formic acid was determined on an aliquot of the volatile acid fraction by the reduction of $HgCl_2$ to Hg_2Cl_2 according to the method of Auerbach and Zeglin (1922), or by oxidation with HgO according to Osburn et al. (1933). In the latter method, the oxidation is carried out in a 300 ml. Erlenmeyer flask connected to a reflux condenser and then to a bead tower containing fifteen ml. of CO_2-free NaOH. The whole system is placed under a slight negative pressure. Formic acid was oxidized to CO_2 and the CO_2 was collected in the sodium hydroxide bead tower. An aliquot of the NaOH containing the CO_2 from the formic acid was then acidified, and the liberated CO_2 determined manometrically, or gravimetrically by absorption in an ascarite tube.

After oxidation, the HgO was filtered off, the solution neutralized, evaporated to ten or fifteen milliliters, acidified, and fifteen volumes steam distilled. This fraction contained the acetic acid only, the formic acid having been removed, as described above. Acetic acid was identified by the partition method of Osburn et al. (1933) and was quantitatively determined by titration, and by oxidation to CO_2 with persulfate (Osburn and Werkman, 1932). The CO_2 was collected and determined as described above.

Non-volatile acids

The residue of the steam distillation was extracted continuously with ether for twenty-four hours. The ether was

removed by distillation after the addition of a small amount of water. This fraction contained the succinic and lactic acids.

Succinic acid was determined either as the silver salt or with succinic dehydrogenase preparations obtained from beef heart. In the former method, an aliquot of the ether extraction was titrated to a faint pink to phenolphthalein with 0.05 N $Ba(OH)_2$ to remove any phosphates that may have been present. Any precipitate that formed was discarded by filtration. The solution was made just acid to phenolphthalein with 0.1 N HNO_3, and then just alkaline with 0.1 N NH_4OH. Add 5 ml. of 10% $AgNO_3$ solution to the mixture. Allow it to stand for 15 minutes, filter, wash and dry at 110° C for one hour in a Gooch crucible. This procedure also precipitates other dicarboxylic acids, and is therefore not applicable if they are present.

If pyruvic acid is present in large amounts, the silver salt blackens. In this case, succinic acid is usually determined as the barium salt. After the extract is titrated with $Ba(OH)_2$ and filtered if necessary, it is evaporated to 10 ml. and sufficient ethyl alcohol added to make the concentration 85%. The mixture is held in the refrigerator overnight. The precipitate is then filtered and washed with 95% ethyl alcohol, dried, and weighed.

When malic acid is present, its precipitation as the silver salt is not quantitative if the dicarboxylic acid solution is neutralized to phenolphthalein, and then the $AgNO_3$ solution is added. Therefore, in some instances, 5 ml. of 10%

$AgNO_3$ was added to 25 ml. of the dicarboxylic acid solution and the pH adjusted to a purple color to brom-cresol-purple with 0.5 N NH_4OH (Wood et al., 1942). At this reaction the silver salts are quantitatively precipitated.

In pyruvate fermentations with the enzyme preparation, succinic acid was practically the only dicarboxylic acid formed as determined by the use of a succinic dehydrogenase preparation, according to Krebs (1937) and by the use of the homogenizer described by Potter and Elvehjem (1936). Beef heart tissue freed from fat and connective tissue is put through a grinder and then washed in cold water until the washings are colorless. This procedure usually requires seven to eight washings. The tissue is homogenized with a homogenizer made entirely of glass. The end of a glass rod is blown until the bulb formed just fits the inside of a pyrex test tube. The rod is rotated by a motor. Approximately 4 ml. of pH 7.4 phosphate buffer are placed in the test tube plus a small piece of the tissue. The glass pestle is them gently forced up and down in the tube until the tissue is completely homogenized. The homogenized solution is then adjusted to the desired concentration and allowed to stand in the refrigerator for about two hours. The activity of the suspension generally is somewhat increased during this period.

As far as is known, only four substances are oxidized by succinic dehydrogenase preparations: succinate, methyl succinate (Thunberg, 1933), α-glycerophosphate (Green, 1936a),

and d(-) glutamic acid (Weil-Malherbe, 1937). The rates of oxidation of α-glycerophosphate and d(-)glutamic acid are very slow, and neither of these compounds is extracted with ether. The rate of oxygen uptake of succinic dehydrogenase preparations on methyl succinate is about 0.5% to 3% of that of succinate oxidation. Since co-enzymes are not extracted by ether, and since these are not present after the heart muscle is washed, the activity of this preparation is specific for succinate. The succinic dehydrogenase preparation was tested on many different substrates, including ethyl alcohol, acetate, formate, lactate, pyruvate, glycerol, glucose, hexosediphosphate, fumarate and succinate. No activity was observed except with succinate, where there was a quantitative oxygen uptake. For example, 0.2 ml. of 0.1M. succinate (0.02 mM.) in a Warburg cup, plus 0.5 ml. of the succinic dehydrogenase preparation and water to a total volume of two milliliters whould take up 224 µl. of O_2, on the basis that 1 mM. = 22,400 µl. Therefore 0.02 mM. equals 448 µl. of succinate, and 2 mM. of succinic acid take up 1 mM. of oxygen. Controls must be run.

Lactic acid was determined according to Friedemann and Graeser (1933) on the aliquot of the ether extraction, after the silver succinate had been filtered off.

Carbon dioxide

The small amounts of CO_2 evolved during the fermentation of pyruvate were collected in a bead tower containing CO_2-free

NaOH solution, and determined manometrically or gravimetrically, as described above. No H_2 was formed during pyruvate fermentation with this juice, since the small amount of gas formed from pyruvate in the absence of bicarbonate was completely soluble in NaOH.

Carbon recovery and O/R index

The accuracy of the fermentation analyses was checked by the carbon recovery and the O/R index.

The amount of carbon recovered was determined by multiplying the mM. of each product of the fermentation by the number of carbon atoms in each product and adding the values obtained to give the total mM. of carbon in the fermentation products. This total value was then divided by the millimoles of substrate fermented multiplied by its number of carbon atoms. The product obtained, times 100, expresses the per cent of carbon recovered.

Oxidation-reduction indexes were calculated according to Erb, Wood and Werkman (1936). This index is based upon the fact that in an anaerobic experiment, the only source of hydrogen and oxygen, in addition to substrate, is water. These two elements exist in water in a ratio of 2:1. In calculating the results, a molecule of hydrogen is assigned a reduction value of +1; the oxygen atom, an oxidation value of -1. The number of oxygen atoms in a compound, times (-1) plus the number of hydrogen molecules, times (+1), equals the oxidation

(-) or reduction (+) value of the compound. For example, acetic acid ($CH_3.COOH$), lactic acid ($CH_3.CHOH.COOH$) and water (H_2O) are neither oxidized nor reduced. Formic acid (HCOOH), succinic acid ($COOH.CH_2.CH_2.COOH$), and pyruvic acid ($CH_3.CO.COOH$) have oxidation values of one, and CO_2 has an oxidation value of two. Since there is an equivalent reduction for each oxidation, the ratio of hydrogen molecules to oxygen atoms in the final products should be the same as in the original substrate. An O/R value in excess of one indicates a deficiency of reduced products or an excess of oxidized products, and vice versa.

Since pyruvate is an oxidized substance, and all the products formed are neutral or oxidized, the O/R indexes were calculated by multiplying the mM. of each product by its respective oxidation or reduction value, and dividing this total value by the mM. of fermented substrate times its oxidation value, and multiplying by 100.

Dialysis

Dialysis of the enzyme preparation was carried out in a collodion bag against distilled water at approximately 5° C. The dialyzing apparatus* consisted of three parts: (1) a motor rotating a glass rod to which the collodion bag was attached with heavy thread, and sealed off with collodion; the

*Originally constructed by Dr. M. F. Utter.

glass rod extended into (2) the dialyzing chamber at an angle of about 45°; the capacity of the dialyzing chamber was approximately 1500 ml.; it was connected to (3) a reservoir with a 10-liter capacity, containing distilled water and ice cubes. The water was pumped from the dialyzing chamber into the reservoir and allowed to flow back to the dialysis chamber by gravity.

Drying

The bacterial juice was dried by first pouring a small quantity into a petri dish, freezing quickly by putting it in the refrigerator, then evacuating the frozen enzyme preparation in a desiccator containing $CaCl_2$. Without freezing the juice first, the dried product had practically no activity on pyruvate. The dried juice gradually lost almost all of its activity within a period of two months, even though kept at 5° C.

Determination of Oxalacetic Acid

Oxalacetic acid was determined according to the aniline-citrate method of Edson (1935) and the colorimetric method of Straub (1936). In the former method, generally the procedure was as follows. The juice plus buffer and water were placed in the main chamber of the Warburg cup, the substrate in one side arm, and 0.3 ml. of a 50% solution of citric acid in the other side arm. After the substrate had been tipped into the

main chamber and the reaction allowed to proceed, the citric acid was tipped into the main chamber to stop the reaction and liberate all bound CO_2. The manometers were shaken until all the bound CO_2 had been evolved (generally 5 to 15 minutes, depending on the amount of bicarbonate or CO_2 originally present). The manometers were then taken off the bath, and 0.4 ml. of a 1:1 mixture of citrate-aniline quickly added to the side arm originally containing the citric acid. The manometers were then quickly replaced on the bath, the contents of the cups allowed to come to temperature equilibrium (3 to 5 minutes), readings taken, and the citrate-aniline tipped into the main compartment of the cup. The side arm containing aniline is quickly rinsed by the contents of the main chamber. The CO_2 evolved originates from the carboxyl group adjacent to the methylene group of oxalacetic acid. The remainder of the oxalacetate molecule unites with the aniline to form pyruvanilide.

Owing to the general instability of oxalacetate, especially at acid reactions, this determination must be carried out as quickly as possible. However, care must be taken that all the CO_2 is driven off from the medium and the cup has reached temperature equilibrium before the citrate-aniline is tipped into the center well.

Controls are necessary, and if run correctly, generally give slightly negative or zero values for oxalacetate. If the aniline is added to the side arm of the Warburg cup immediately

after the acid is tipped and before all the CO_2 has been evolved, the citrate-aniline solution seems to absorb some CO_2 from the atmosphere, and when tipped into the acid solution in the main chamber, CO_2 is evolved. If the controls give a positive result, the test is worthless because of the small amounts of oxalacetate formed from pyruvate. However, if the aniline is added after all the CO_2 has been liberated from the medium, the test can be adapted to detect small quantities of oxalacetic acid.

Straub's colorimetric method was used to check the formation of oxalacetate from pyruvate and CO_2. The reagents employed were (1) a hydrazine solution made up by dissolving 3.5 grams hydrazine hydrochloride in 30 ml. water, then adding 100 ml. of 95% ethyl alcohol; (2) a saturated $NaNO_2$ solution; (3) a KOH solution made up by dissolving 100 grams KOH in 60 ml. water. The contents of a Warburg cup (two milliliters) were acidified with 0.3 ml. of a 10% solution of trichloracetic acid. The solution was then quickly filtered through a Whatman 42 filter, into a test tube containing 1.4 ml. of hydrazine reagent. A little suction was applied to speed up the process and to obtain as much as possible of the original cup contents for the test. Generally, about 1.8 to 1.9 ml. of liquid was obtained from the original cup contents of 2.3 ml. After filtering into the hydrazine, the mixture was shaken, warmed at 37° C for fifteen minutes and cooled in ice water for three minutes. Then 0.1 ml. of $NaNO_2$ solution was added,

the mixture shaken, and five minutes later, one ml. of KOH solution was added. If oxalacetate is present, a yellow color develops. The contents of the test tubes were read in a Klett-Summerson photoelectric colorimeter with the 420 mμ. filter. Known amounts of oxalacetate were run, and the values plotted in a curve. Control cups had exactly the same contents as the experimental cups, but the pyruvate and acid were tipped simultaneously into the center well containing the juice. The controls had a small blank reading, due to the retention of some of the original color of the juice. Control values were subtracted from those of the experimental cups.

Preparation of Heavy-Carbon Bicarbonate* and Acetate*

$C^{13}O_2$ was obtained by burning methane whose C^{13} content had been concentrated in a thermal diffusion column according to Nier and Bardeen (1941). The $C^{13}O_2$ was collected in NaOH and then precipitated as $BaC^{13}O_3$. The $BaC^{13}O_3$ was acidified with 5 N H_2SO_4 in a closed system under a vacuum, and the $C^{13}O_2$ was collected in an equivalent amount of N carbonate-free NaOH.

Acetic acid containing an excess of C^{13} in the carboxyl group was prepared according to the following reactions:

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*Prepared by Dr. H. G. Wood.

$$CH_3I + Mg \longrightarrow CH_3.MgI$$

$$CH_3MgI + C^{13}O_2 \longrightarrow CH_3.C^{13}OOMgI$$

$$CH_3.C^{13}OOMgI + H_2O \longrightarrow CH_3.C^{13}OOH + MgIOH$$

Determination of C^{13} Content of Fermentation Products

In order to determine the C^{13} content of the compounds isolated from fermentations to which the isotope was added in the form of $NaHC^{13}O_3$ or $CH_3.C^{13}OOH$, it was necessary to convert these compounds to CO_2.

Formic acid was oxidized to CO_2 with HgO. (Osburn et al., 1933). Acetic acid was oxidized to CO_2 with persulfate, according to Osburn and Werkman (1932). Succinate was converted to a mixture of fumarate and malate by means of a succinic dehydrogenase preparation. The malate was then oxidized with $KMnO_4$ to acetaldehyde which was collected in a bisulfite bead tower, plus 2 CO_2 collected in an NaOH bead tower. The two carbon dioxide molecules originate from the two carboxyl groups, and acetaldehyde from the methylene groups of the original succinic acid (Wood et al., 1941b). The acetaldehyde was further oxidized to CO_2 with potassium persulfate (Osburn and Werkman, 1932). Lactic acid was oxidized with $KMnO_4$ (Friedeman and Graesser, 1933), which converted the carboxyl group to carbon dioxide, and the rest of the molecule to acetaldehyde. The carbon dioxide and acetaldehyde were collected as described above. The acetaldehyde was further oxidized to carbon dioxide with persulfate.

The C^{13} content of the carbon dioxide samples was determined by mass spectrometer analysis, according to Nier (1940).

EXPERIMENTAL

Factors Affecting Preparation of an Active Bacterial Enzyme System

Bacterial enzyme preparations were obtained in this laboratory according to a method developed by Wiggert *et al.* (1940). Bacterial cells were mixed with finely ground pyrex glass, ground in an iced mortar and pestle, and extracted with phosphate buffer. In attempts to improve the method, the effect of grinding time, grinding agents, and ratios of cells to grinding agent on the production of an active bacterial juice was studied.

Grinding time and grinding agents

Experiments were conducted to determine the comparative values of glass and carborundum (Alloxite brand powder, No. 1000 and finer) as grinding agents in obtaining active preparations from *E. coli*. The cells were grown in five liters of glucose broth, and the activities of their enzyme preparations were tested on glucose and hexosediphosphate. A summary of some of those results is presented in Table 1. It can be seen that generally glass is superior to carborundum as a grinding agent under the conditions of the experiment. Grinding the cells for one minute did not result in as active a juice as when the

cells were ground for three minutes. However, grinding for five minutes seemed to destroy some of the activity of the preparation.

Table 1. Effect of grinding time and different grinding agents on activity of juice obtained from E. coli.

Grinding agent	Ratio* of cells to grinding agents	Grinding time (min.)	μl. CO_2 evolved per hour	Average μl. CO_2 per hr.
glass	1:8	1	164 165 162	164
		3	221 239 278	246
		5	167 217	192
carborundum	1:8	1	98 102 104	101
		3	84	84
		5	77 88	83

*Ratio according to weight (grams).
Each cup contained: bacterial juice (in side arm), 0.6 ml.; glucose, 0.04 M.; hexosediphosphate (1.25%), 0.4 ml.; $NaHCO_3$, 0.06 M.; total volume, 2.0 ml.; atmosphere, CO_2; temperature, 30.4° C; time, one hour; substrates previously saturated with CO_2.

Ratio of cells to grinding agents

We were not sure that the grinding was being carried out under optimum conditions. Perhaps, if conditions were changed

carborundum might be superior. Therefore, varying ratios of cells to glass and cells to carborundum were mixed and ground to see what effect different experimental conditions might have on the activity of the enzyme preparations obtained. More active preparations (Table 2) were obtained when the ratio of cells to grinding agent was reduced from 1:8 to 1:3, for both glass and carborundum. Glass was still a superior grinding agent. In another experiment, further comparison was made between 1:3 and 1:2 cell-glass ratios. The 1:2 ratio yielded a slightly more active juice (Table 2). In this instance, harder grinding probably produced a more active preparation, compared to previous results. It would seem that the presence of more glass would give better grinding results and more active preparations, but, experimentally, the opposite was true.

Cell counts of enzyme preparations

Inasmuch as the enzyme preparation was not actually free of cells, the next step was to determine what part of the activity of the bacterial juice was due to the presence of living bacterial cells. Previously, it had been determined (Utter, 1942) that approximately 45 million cells had to be added to a juice before an appreciable increase in activity could be noted on the respirometer. The effect observed under these conditions increased progressively. This increase was not observed with the bacterial juice.

Using suitable dilutions, and aseptic techniques, a series of plating experiments were carried out with several

Table 2. Effect of varying ratios of cells to grinding agents on activity of enzyme preparation obtained.

Grinding agent	Ratio--cells: grinding agent	μl. CO_2 evolved per hour	Average μl. CO_2 per hour
carborundum	1:1	99	
		84	92
	1:3	216	
		233	
		274	241
	1:5	99	
		113	
		151	121
	1:8	84	84
glass	1:3	331	
		393	
		410	378
	1:5	265	
		267	
		283	272
	1:8	234	
		259	
		277	257
glass	1:3	520	
		504	512
	1:2	553	
		551	552

NOTE: Each cup contained: juice, 0.6 ml.; glucose, 0.04 M.; hexosediphosphate (2.5%), 0.2 ml.; $NaHCO_3$, 0.06 M.; total volume, 2 ml.; atmosphere, CO_2; time, 1 hour; temperature, 30.4° C. Substrates previously saturated with CO_2. Grinding time, 3 minutes.

bacterial enzyme preparations. The petri dishes contained nutrient agar, and were read at 24- and 36-hour intervals. It was found that the bacterial juices ordinarily contained 50,000 to 500,000 organisms per milliliter. According to the results obtained by Utter (1942), this number was not enough to appreciably affect the activity of the juice.

One cell-glass mixture was ground in 10-gram batches, and two other mixtures were ground in 8-gram batches. The glass was then centrifuged off, and the preparations were given various treatments on the Beams ultracentrifuge. The juices were all centrifuged on the Beams in the large cup for six minutes at 64 pounds air pressure. The resulting liquids were not quite clear. They were frozen and again centrifuged for six minutes. One milliliter of the clear liquid was plated and 0.8 ml. tested for activity on the respirometer. Half of one preparation was centrifuged a third time on the Beams in the small cup, for two minutes at 64 pounds pressure and also was plated and tested for activity. The results are presented in Table 3. It was found that when the cell-glass mixture was ground in 10-gram batches, the grinding process was not so efficient as when the mixture was ground in 8-gram batches. The former preparation had an average cell count per milliliter of 330,000 to 450,000, while the count for the latter was 20,000 to 40,000 organisms per milliliter. There was only a slight difference in the activity of the two preparations. The preparation which was centrifuged a third time had a very

Table 3. Effect of cell count on activity of enzyme preparation obtained from E. coli.

Treatment of juice	Activity (μl. CO_2/hr.)	Average μl. CO_2/hr.	Organisms per ml. of juice
*	1010		
	1100		330,000-
	1060	1060	450,000
**	936		
	1028		
	1012	992	20,000-
			40,000
***	868		3,000-
	827	847	30,000

*Cell-glass mixture ground in 10-gram batches; 4.5 ml. centrifuged on Beams for six minutes at 64 pounds pressure; frozen, centrifuging repeated.

**Ground in 8-gram batches. Same centrifuging treatment as in (*) above.

***Same treatment as (**), plus a third centrifugation: 2 ml. of juice centrifuged in small cup for two minutes at 64 pounds pressure.

NOTE: Each cup contained: bacterial juice, 0.6 ml.; glucose, 0.04 M.; HOP (2.5%), 0.2 ml.; $NaHCO_3$, 0.06 M.; total volume, 2.0 ml.; atmosphere, CO_2; time, 1 hr.; temperature, 30.4° C.

low cell count (3,000 to 30,000), and its activity was decreased from approximately 990 to 850 μl. CO_2 per hour, as tested on glucose and hexosediphosphate. Thus it was determined that the activity of the enzyme preparation was due to the cell-free material present, and that with careful handling the cell count of the juice could be kept well below 50,000 cells per milliliter without affecting its activity.

The next phase of the problem was the preparation of a bacterial enzyme system which would utilize CO_2. From previous work in this laboratory, it was shown that CO_2-utilization was connected with succinic acid formation (Wood and Werkman, 1938). Therefore, could we obtain a bacterial juice which would form succinic acid?

Effect of growth media

It has been shown that E. coli can utilize CO_2 and form succinic acid. It is also known that coli organisms will form large amounts of succinic acid from galactose or pyruvate, but very little from glucose (Krebs, 1937). The enzyme preparation described above prepared from cells grown in glucose broth was active on glucose but was found to be inactive on galactose or pyruvate. Preparations obtained from cells grown in glucose and galactose broth culture were still very active on glucose but only slightly active on galactose. When the cells were grown in galactose broth culture, the resulting preparation was inactive on glucose or galactose.

Since an active enzyme preparation which would ferment galactose or pyruvate (and produce succinate) could not be obtained from cells grown in a glucose medium, further experiments were designed with two purposes in mind: first, to determine the amounts of succinic acid produced by E. coli cells grown in various media, and secondly, to determine which of these batches of cells would lend itself to grinding and to the production of active juices.

It can be seen from the results in Table 4 that cells grown in different media form different amounts of succinic acid from pyruvate. Thus cells grown on an agar-beef extract medium form the largest amounts of succinic acid. The addition of glucose to this medium increases the yield of cells but decreases the resulting succinic acid formed by these cells from pyruvate. Cells grown in glucose medium form very small amounts of succinic acid. When the glucose medium was kept alkaline at all times, the succinate did not increase. Thus, the agar-beef extract medium, originally described by Krebs (1937), seemed most favorable for the production of cells which would form relatively large amounts of succinic acid. Attempts were made to obtain an enzyme preparation from cells grown on this medium, but the cells seemed more resistant to grinding than the cells grown in glucose broth culture. The resulting juice was very thin, and had only slight activity on glucose, galactose, and pyruvate (approximately 30 μl. CO_2 evolved per hour). However, this was the first indication of a juice obtained from E. coli which had any activity at all on pyruvate.

The liquid beef extract medium without aeration was not practicable, since it yielded only 4 to 6 grams of cells per 10 liters. Aeration increased the yield of cells to 15 to 25 grams, and the resulting cells still formed appreciable quantities of succinic acid from pyruvate (Table 4). A juice was prepared from E. coli cells grown in this aerated liquid beef

Table 4. Variation in amounts of succinic acid produced by *E. coli* grown in various media.

Medium	mM Succinic acid per 100 mM Pyruvic acid fermented
1. Agar, beef extract	19.2
2. Liquid beef extract	12.6
3. Liquid beef extract plus aeration	11.5
4. No. 3 plus glucose	8.9
5. No. 3 plus glucose, kept alkaline	3.4
6. Glucose	4.2

Media:

1. Beef extract 1.0%, Yeast " 0.5, Peptone 1.0, NaCl 0.5, Agar 3.0, Tap H_2O 10.0

2. Beef extract 1.0%, Peptone 0.4, Yeast extract 0.5, NaCl 0.5, Tap H_2O 10.0

4. Same medium as No. 3, plus 1% glucose.

6. Glucose 1.0%, Peptone 0.4, Distilled H_2O 90.0; K_2HPO_4 0.8%, Tap H_2O 10.0. Sterilized separately.

extract medium (No. 3, Table 4). The juice was inactive on glucose or galactose plus hexosediphosphate, but was extremely active on pyruvate, 0.8 ml. of the juice liberating over 1000 μl. of CO_2 within two hours (Table 5). The contents of three of the Warburg vessels were then acidified, to precipitate the proteins and to stop the reaction, and carefully

Table 5. Dissililation of pyruvate and formation of succinic acid by enzyme preparation of Escherichia coli.

μl. CO_2 Evolved	mM Succinic acid formed per 100 mM of pyruvic acid fermented
1008	
1150	
1200	6.07 (succinic dehydrogenase method)
1130	6.88 (silver salt method)
1100	

NOTE: Each cup contained: pyruvate, 0.044 M; $NaHCO_3$, 0.038 M; juice, 0.8 ml.; total volume, 2.0 ml; atmosphere, CO_2; time, two hours; temperature, 30.4° C.

rinsed out. The fermentation liquid was then steam distilled to remove volatile acids, and the residue extracted with ether over night. The ether extract was tested for the presence of succinic acid by two methods. The first one employed an enzyme, succinic dehydrogenase, specific for succinic acid to quantitatively determine the amount of succinate present by the corresponding uptake of oxygen in the conversion of the succinate to fumarate. In the second method, the succinic acid was precipitated as its silver salt, and determined gravimetrically. Both methods showed that succinic acid was present (Table 5).

Thus, an enzyme system had been prepared which fermented pyruvate and produced succinic acid. The next phase of this investigation was, therefore, devoted to studying the

properties and components of the enzyme preparation in the anaerobic dissimilation of pyruvate.

Summary and conclusions

Glass, generally, is superior to carbonundum as a grinding agent for obtaining active enzyme preparations from bacteria. Optimum grinding times were determined, and it was found that extended grinding periods resulted in weaker preparations. The mixing of large amounts of ground glass with cells is not necessary for production of active juices. In fact, when the ratio of cells to glass by weight was lowered from 1:8 to the optimum of 1:2, more active preparations were obtained. By careful handling, the cell count of the preparation can be kept well below 50,000 bacteria per milliliter without affecting its activity. It was also demonstrated that the activity of the enzyme system was not due to the small amounts of cells present, but to the cell-free enzymes present in the preparation. The effect of different growth media on the production of succinic acid from pyruvate by *E. coli* cells and on the preparation of active juices from these cells was demonstrated. An active enzyme preparation was obtained, capable of fermenting pyruvate, with the production of succinic acid.

Anaerobic Dissimilation of Pyruvate

Previous investigations on pyruvate with cell-free enzyme preparations from bacteria have been largely limited to studies of the aerobic dissimilation. More recently, Silverman and Werkman (1941) and Koepsell and Johnson (1942) have investigated the anaerobic dissimilation of pyruvate with a cell-free preparation. Silverman and Werkman (1941) described an enzyme preparation obtained from Aerobacter by the glass-grinding method, which converted pyruvate into acetylmethylcarbinol and CO_2. Koepsell and Johnson (1942) described an enzyme preparation obtained from Clostridium butylicum by alternate freezing and thawing, which converted pyruvate into acetic acid, CO_2 and molecular hydrogen.

The aerobic investigations are those chiefly of Lipmann (1939 for summary) and Still (1941). Lipman obtained extracts of dried cells of Lactobacillus delbrueckii which oxidized pyruvate to acetate and CO_2. Still, using the Booth-Green mill, obtained a similar pyruvic dehydrogenase system from Escherichia coli. Both preparations, however, had almost no activity under anaerobic conditions. Therefore, it is the purpose of this part of the investigation to compare the mechanism of anaerobic dissimilation of pyruvate by the E. coli enzyme preparation with the mechanism of aerobic oxidation of pyruvate, as performed by extracts of L. delbrueckii and E. coli. In addition to the pyruvate enzyme, the preparation

reported here contains very active formic dehydrogenase and hydrogenase activity.

Optimal enzyme and substrate concentrations

The enzyme is active on pyruvate anaerobically and liberates a large amount of carbon dioxide in bicarbonate buffer. In order to determine the effect of enzyme concentration on the dissimilation of pyruvate (Table 6), amounts of juice varying from 0.4 ml. to 1.0 ml. were added to a series of Warburg cups whose contents were identical in all other respects. A very striking dilution effect was observed; i.e., there was little activity with 0.5 ml. of the juice (80 μl. evolved), whereas with addition of 0.6 ml. of the juice, the CO_2 evolution increased from 80 μl. to 862 μl. The optimal amount of juice was found to be 0.8 ml., with no further increase in CO_2 evolution on addition of larger amounts of

Table 6. Effect of enzyme concentration on dissimilation of pyruvate.

Juice (ml.)	CO_2 Evolved (μl.)
0.4	17
0.5	80
0.6	862
0.8	931
0.9	881
1.0	958

NOTE: Each cup contained: juice in indicated amounts; pyruvate, 0.035 M; $NaHCO_3$, 0.038 M; total volume, two milliliters; atmosphere, CO_2; time, three hours. Temperature, 30.4° C.

the enzyme. Most of the CO_2 was evolved during the first hour and a half, but the experiment was allowed to proceed for three hours in order to determine whether there was an actual dilution effect, or whether with smaller amounts of enzyme, the rate of CO_2 evolution was just slower.

More than 1000 μl. of carbon dioxide are evolved in one hour by 0.8 ml. of the enzyme; the values depend on the amount of pyruvate present (Table 7), which is never completely fermented even in low concentrations. The limiting factor is the biological availability of the substrate. The enzyme system is easily saturated since concentrations of pyruvate beyond 0.03 M have no appreciable effect on the rate of CO_2 evolved.

Effect of pH on enzyme activity

The ground cells were extracted with water, and phosphate buffer of a definite pH was added to each cup. After two hours the contents were deproteinized with trichloracetic acid and diluted to a convenient volume. Residual pyruvate was determined on an aliquot containing less than 0.5 mg. per ml. The dissimilation of pyruvate (Table 8) proceeds within a range of approximately pH 5.8 to 8.0; the rate shows a marked change with pH between pH 6.2 and 7.2 with the optimum near pH 6.8. In contrast to these results, the optimum pH of the aerobic mechanism of E. coli studied by Still was 6.17, with practically no activity at pH 7.0. The optimum for the aerobic mechanism of L. delbrueckii (Lipmann, 1939) was between 6.0 and 6.5, and that for Cl. butylicum (Koepsell and

Table 7. Effect of substrate concentration on enzyme saturation in dissimilation of pyruvate.

Cup no.	Pyruvate concentration (M)	Per cent fermented	μl. CO_2 evolved	
			1 hr.	15 min.
1	0.015	90.1	301	256
2	0.030	91.7	530	318
3	0.045	91.2	812	334
4	0.060	85.7	828	
5	0.075	70.1	951	320
6	0.090	63.9	1076	359

NOTE: Each cup contained: enzyme, 0.8 ml.; $NaHCO_3$, 0.045 M; (No. 5 and No. 6, 0.06 M $NaHCO_3$); pyruvate, in indicated concentrations. Total volume, 2 ml. Atmosphere, 10% CO_2 in N_2. Temp., 30.4° C.

Table 8. Effect of pH on the dissimilation of pyruvate by the enzyme preparation of E. coli.

pH	5.4	5.8	6.2	6.4	6.77	6.94	7.2	7.3	7.5	7.9
Pyruvate fermented (mgs.)	0.0	0.42	3.38	4.01	4.33	5.96	2.37	0.90	0.85	0.42
% Pyruvate fermented	0	8	64	76	82	75	44	17	16	8

NOTE: Each cup contained: enzyme, 0.6 ml.; pyruvate, 5.28 mgs./2 ml. (0.03 M); phosphate buffer (0.25 M). Total volume, 2 ml. Time, 2 hrs. Atmos., N_2. Temp. 30.4° C.

Johnson, 1942) was 6.5. The preparation used in this investigation retains almost 50% of its activity at pH 7.2, whereas below 6.2 and above 7.2 there is a sharp decrease in the pyruvate utilized.

Effect of temperature

The preparation is not affected by temperatures up to 40° C for five minutes (Table 9). When heated at 43° C for five minutes, it becomes translucent, but no inactivation occurs. At 47° C some of the proteins precipitate, and the solution becomes opaque; but no loss in activity occurs, as judged by the pyruvate utilized. At 50° C the preparation still retains the greater part of its activity, and inactivation is only 6 per cent. From 50° to 55° C inactivation gradually increases to 87 per cent, and between 55° and 57° C activity is completely destroyed.

The preparation obtained by Silverman and Werkman (1941) from Aerobacter retained 6 to 7 per cent of its activity on pyruvate when heated for five minutes at 65° C. This stability correlates with the results of Melnick and Stern (1940) who found that five minutes at 60° C reduced the activity of yeast carboxylase to 4 per cent. The present preparation is less stable to heat than those obtained from Aerobacter and yeast and is comparable to Still's aerobic pyruvic dehydrogenase preparation which is inactivated when heated to 55-60° C.

Effect of drying

The clear liquid was tested for activity, then frozen and dried over night in a vacuum desiccator. The dried material was then ground gently with a mortar and pestle and resuspended in its original volume of distilled water. There was no loss of activity in preparations tested after a few days.

Table 9. Effect of temperature on activity of the enzyme preparation.

Temperature (5 min.)	Pyruvate fermented (mgs.)	CO_2 evolved (μl.)	% Inactivation
30° C	8.99	729	0
40° C	8.99	714	0
43° C	9.00	746	0
47° C	9.09	690	0
50° C	8.46	594	6.2
52° C	7.49	533	17
53° C	4.18	332	54
55° C	1.19	-49	87
57° C	0.00	-56	100
Control (no enzyme)	0.00	-1	--

NOTE: Enzyme heated for five minutes. Each cup contained: enzyme, 0.8 ml.; pyruvate, 10.56 mgs. (0.06 M); $NaHCO_3$ (0.045 M). Total volume in each cup, 2.0 ml. Atmos., 10% CO_2 in H_2. Time, 1 hr. Temp., 30.4° C.

For example, 778 μl. CO_2 were liberated by the liquid in one hour, and 799 μl. CO_2 by the dried preparation. One batch of dried material was allowed to remain at 5° C for 53 days with a resulting sharp decrease in activity as tested manometrically (99 μl.). When the preparation was dried without first being frozen, it was completely inactivated.

Formic dehydrogenase and hydrogenase activity

The enzyme preparation contains very strong formic dehydrogenase activity and reduces methylene blue in less than two minutes with formic acid as the hydrogen donator (Table 10). Stickland (1929) obtained a cell-free enzyme preparation from E. coli through tryptic digestion, containing the enzyme, formic dehydrogenase. Gale (1939) by the use of the Booth-Green mill obtained this same enzyme. In neither case, however, was the active formic dehydrogenase separated from the solid particles. The enzyme preparation used in this investigation is a clear liquid, and was obtained by removing cells and debris in a Beams ultracentrifuge (capacity, 5 ml.) at approximately 100,000 r.p.m. for 10 minutes. The activity of the formic dehydrogenase thus does not seem to be bound to the solid particles.

The enzyme hydrogenase, which reduces methylene blue with gaseous hydrogen as the only hydrogen donator, is also present in a very active form (Table 10). All decolorization measurements were taken as the time necessary for complete reduction of methylene blue.

Both of these enzymes, as they are present in this preparation, are quite stable. They can be reduced to a powder by freezing and drying in vacuo, and resuspended in water with no appreciable loss in activity. After having remained dried for almost two months, the enzymes still retained the greater part of their activity (Table 10). Lee et al. (1942) have

Table 10. Formic dehydrogenase and hydrogenase activity of E. coli enzyme preparation.

Enzyme	Treatment		
	None	Dried (53 days)	Controls (no substrate)
Formic dehydrogenase	Min.* 1.75	Min. < 5	Min. > 60
Hydrogenase	1	7	> 60

*To decolorize methylene blue.

NOTE: Each cup contained: enzyme, 0.8 ml.; $NaHCO_3$, 0.045 M; methylene blue (1/14,000). Substrate for formic dehydrogenase was 0.5 ml. 0.1 M HCOOH. Atmos., N_2. Substrate for hydrogenase, H_2. Total volume, 2.8 ml.

obtained active hydrogenase preparations from Azotobacter, and Bovarnick (1941) by acetone treatment of E. coli has obtained cell-free powders containing active hydrogenase.

Aeration during growth destroys formic hydrogenlyase (Yudkin, 1932). Bacteria used in the present experiments were grown under aeration and did not contain that enzyme; i.e., formic acid was not broken down to H_2 and CO_2, even after 24 hours' incubation. The fact that both formic dehydrogenase and hydrogenase are present, but not formic hydrogenlyase, disposes of the possibility that the absence of formic hydrogenlyase may be due to separation of these two enzymes within the intact cell.

Products of pyruvate dissimilation

Pyruvate is attacked anaerobically to form CO_2, acetic,

formic, and succinic acids, and a trace of lactic acid. No H_2 is formed. Aerobically, oxygen is not taken up, although the pyruvate is fermented. Under an atmosphere of N_2 and with no bicarbonate present in the medium, very little succinic acid is formed. The effect of different gases on succinate formation was investigated, and it was found that the yield of succinate was greatest under an atmosphere of 5 to 10 per cent CO_2 in H_2. This gas mixture was obtained in experiments 2, 3, and 4 (Table 11) by the addition of acid phosphate to a medium which contained bicarbonate and was under an atmosphere of H_2. Experiment 1 was carried out in a large cup on the respirometer, whereas the others were carried out in a stationary 300 ml. Ehrlenmeyer flask connected to a NaOH bead tower. The fermentation was stopped by addition of sulfuric or metaphosphoric acid. Carbon balances and O/R indexes were determined in order to check the accuracy of the analyses. A small error in the determination of a strongly oxidized product (for example CO_2) will result in a large error in the O/R index, which is the case in Experiment 2.

Components of the enzyme system

The enzyme system is easily inactivated by dialysis for half an hour and reactivated on the addition of phosphate (Table 12). The necessity of phosphate in the oxidation of pyruvate has been shown by Virtanen and Karström (1931), Lipmann (1939), Banga et al. (1939), and Still (1941). Silverman and Werkman (1941) showed phosphate was necessary

Table 11. Products of pyruvate dissimilation by *E. coli* enzyme preparation.

	Experiment No.			
	1	2	3	4
Pyruvate fermented (mM)	2.59	2.52	2.85	2.67
Products per 100 mM of pyruvate fermented:				
CO_2 (mM)	5.65	5.2	7.4	6.38
Formic acid (mM)	76.81	62.0	80.7	69.3
Acetic acid (mM)	88.62	74.4	80.7	76.1
Lactic acid (mM)	3.32	2.8	2.5	3.75
Succinic acid (mM)	4.71	12.8	8.4	14.36
Carbon recovery, %	96	92	96	99
O/R index	0.93	0.84	1.03	0.97
Atmosphere	N_2	CO_2 in H_2	CO_2 in H_2	CO_2 in H_2

NOTE: Experiment No. 1 contained: 18 ml. juice; pyruvate (0.12 M). Total volume, 30 ml. Time, 2 hrs.
Experiment No. 2 contained: 23 ml. juice; pyruvate (0.04 M); $NaHCO_3$ (0.04 M); phosphate, 5 ml. (0.1 M; pH, 6.2). Total volume, 70 ml. Time, 4 hrs.
Experiment No. 3 contained: 30 ml. juice; pyruvate (0.05 M); $NaHCO_3$ (0.05 M); phosphate, 7 ml. (0.1 M). Total volume, 75 ml. Time, 4 hrs.
Experiment No. 4 contained: 38 ml. juice; pyruvate (0.04 M); $NaHCO_3$ (0.07 M); phosphate, 10 ml. Total volume, 75 ml. Time, 4 hrs.

Table 12. Effect of phosphate on pyruvate dissimilation by dialyzed E. coli juice.

Experiment No.	Dialyzing time (mins.)	Ml. CO_2 evolved, 1 hr.	
		Dialyzed juice	Dialyzed juice + PO_4
1	25	203	745
2	30	147	586
3	35	0	560
4	45	109	530
5	90	17	21

NOTE: Each cup contained: dialyzed juice, 1.0 ml.; pyruvate (0.045 M); $NaHCO_3$ (0.045 M); phosphate (pH, 6.88), 0.02 M; water to 2.0 ml. Atmosphere, 10% CO_2 in H_2. Time, 1 hr.

for the anaerobic conversion of pyruvate to acetylmethylcarbinol, and Koepsell and Johnson (1942) also demonstrated the necessity of phosphate in the evolution of H_2 from pyruvate anaerobically. Thus, phosphate is also necessary for the breakdown of pyruvate to acetic and formic acids via the hydroclastic reaction. Concentrations of phosphate up to 0.01 M (Table 13) have no appreciable effect on the dissimilation of pyruvate. There is only a slight effect when the phosphate concentration is increased to 0.015 M, and a decided effect at 0.018 M, above which more phosphate does not appreciably affect the pyruvate breakdown. These results agree with those of Lipmann, and Koepsell and Johnson, who reported optimal

Table 13. Effect of phosphate concentration on pyruvate dissimilation by dialyzed E. coli juice.

Conc. of PO_4	μl. CO_2 evolved	Conc. of PO_4	μl. CO_2 evolved
--	11	0.013	107
0.003	17	0.015	89
0.005	26	0.018	507
0.008	61	0.02	434
0.01	32	0.025	536

NOTE: Juice dialyzed 37 minutes. Each cup contained: dialyzed juice, 1.0 ml.; pyruvate (0.045 M); $NaHCO_3$ (0.045 M) + phosphate in indicated concentrations. Total volume of each cup, 2.0 ml. Atmos., 10% CO_2 in H_2. Time, 2.5 hrs.

phosphate concentrations of 0.015 M and 0.02 M for pyruvate breakdown; below these concentrations, the rates of these two systems were proportional to the phosphate concentrations. The pH of the medium was checked on the Coleman pH apparatus before and after the addition of phosphate, and it was determined that the effect observed was due to the phosphate and not to any change in pH caused by the addition of the phosphate.

In order to demonstrate the necessity of manganese and cocarboxylase in the system, the enzyme preparation had to undergo extensive dialysis, during which it lost a large part of its activity. After 100 minutes' dialysis, the addition of phosphate and cocarboxylase markedly stimulated the reaction,

whereas the addition of Mn had a somewhat smaller effect (Table 14), probably because the manganese is closely bound in the system and is difficult to remove completely by dialysis. The necessity of Mn^{++} and cocarboxylase in anaerobic pyruvate breakdown correlates with their requirement in the aerobic oxidation of pyruvate by brain tissue (Ochoa, 1939b, and Banga et al., 1939), and the aerobic oxidation of pyruvate by bacteria (Lipmann, 1939, and Still, 1941).

Table 14. Effect of phosphate, cocarboxylase and manganese on pyruvate dissimilation by dialyzed juice of E. coli.

	Additions to manometer cups (ml.)				
Juice	1.0	1.0	1.0	1.0	1.0
Pyruvate (0.3 M)	0.3	0.3	0.3	0.3	0.3
$NaHCO_3$ (0.3 M)	0.3	0.3	0.3	0.3	0.3
Phosphate (0.2 M; pH, 6.88)	--	0.2	--	0.2	0.2
Cocarboxylase (150 μg./ml.)	--	0.1	0.1	--	0.1
Mn (0.05 M)	--	0.1	0.1	0.1	--
H_2O	0.4	--	0.2	0.1	0.1
1. CO_2, 1 hr.	-14	104	-14	16	54

NOTE: Juice dialyzed 100 minutes. Atmos., 10% CO_2 in H_2.

Mg^{++} was not as effective as Mn^{++} during the first hour (Table 15). However, at the end of three hours, the effects of Mn^{++} and Mg^{++} were quite similar.

Table 15. Comparison of Mn and Mg effects on pyruvate dissimilation by dialyzed E. coli juice.

Cup contents	1 hr.	2 hrs.	3 hrs.
Dialyzed juice + cocarboxylase, PO_4, Mn	645	1123	1281
Dialyzed juice + cocarboxylase, PO_4, Mg	37	325	1054
Dialyzed juice	-37	40	160

NOTE: Juice dialyzed 36 minutes. Cups contained: dialyzed juice, 1.0 ml.; pyruvate (0.045 M); $NaHCO_3$ (0.045 M); phosphate buffer (0.02 M; pH, 6.88); cocarboxylase, 15 μg.; Mn or Mg (0.005 M). Total volume, 2.3 ml. Atmos., 10% CO_2 in H_2.

Lipmann (1939) reported flavin-adenine-dinucleotide to be a component in the dismutation of pyruvate by extracts of L. delbrueckii and Still (1941) found slight increases in pyruvate oxidation by E. coli juice on addition of the flavin compound. Under the conditions of our experiments, addition of flavin-adenine-dinucleotide was not effective.

After dialysis for one and one-half to two hours, the pyruvate system was inactivated, and activity was not restored on addition of phosphate, Mn^{++} and cocarboxylase. Further addition of co-enzyme I, adenylic acid, adenosinetriphosphate, Mg^{++}, riboflavin, fumarate, Ca pantothenate or biotin, in various combinations failed to restore the activity of the dialyzed enzyme preparation, whereas the addition of boiled

yeast juice gave a stimulation. It is possible that some other component besides phosphate, Mn^{++}, cocarboxylase, and protein, is a part of this system.

In this connection, many experiments were carried out with pantothenic acid and biotin in attempts to determine whether these substances were possible components of the E. coli enzyme system functioning in pyruvate metabolism. It is known that both pantothenic acid and biotin are necessary growth factors for a large number of microorganisms and recent investigations have indicated some possible functions of these two vitamins in carbohydrate utilization. As far back as 1936, Williams et al. found that pantothenic acid was important in respiration, fermentation and glycogen storage by yeast. Further work by Pratt and Williams (1939) showed that pantothenic acid stimulated respiration of yeast, and fermentation by yeast maceration juice. According to Burke et al. (1941), biotin stimulates fermentation and respiration of yeast from a biotin-deficient medium. Further evidence of a possible functional relationship of biotin and pantothenic acid was presented by Wilson and West (1939). They showed that Rhizobium required biotin for maximum development, and that pantothenic acid was stimulatory in the presence of biotin concentrates, although inactive alone. Pantothenic acid was shown to be linked to vitamin B_1 in its effect on yeast growth by the work of Williams and Saunders (1934).

More recent investigations seem to show that pantothenic

acid may be connected with pyruvate utilization. Dorfman et al. (1942) showed that addition of pantothenic acid increases pyruvate oxidation by Proteus morganii. Pilgrim et al. (1942) demonstrated that liver from pantothenic acid- and biotin-starved rats has a decreased rate of pyruvate oxidation. These results may indicate either that pantothenic acid or biotin, or both, may be necessary for pyruvate oxidation, or may be necessary only during formation of the enzyme system which oxidizes pyruvate. However, recent work by Teague and Williams (1942) has shown that pantothenic acid has no effect on glucose dissimilation or pyruvate decarboxylation by a Lebedev juice obtained from yeast.

Since it was determined that some other component besides phosphate, manganese, cocarboxylase, and protein was a part of the enzyme system of E. coli responsible for the dissimilation of pyruvate, investigations were conducted to determine whether pantothenic acid or biotin restores the activity of dialyzed enzyme preparations. In several experiments, definite effects were observed for pantothenic acid and biotin (Table 16), and in many other experiments these results could not be duplicated. In experiment number one (Table 16), the components added to the dialyzed juice showed no effect; i.e., there was no evolution of CO_2 within two hours. Further readings were taken after 4-1/2 hours, when the effects were noted. However, growth could have taken place during that time, and in view of this possibility, the value of the

results is questionable. In experiment 2 the readings were taken after 20 minutes. After 30 minutes the effects had almost disappeared. In experiment 3, a much greater stimulation was obtained on addition of cocarboxylase and manganese than on addition of ca-pantothenate or biotin, or both. In this experiment, the addition of calcium pantothenate or biotin, or both, to the dialyzed preparations already containing phosphate, cocarboxylase and Mn^{++}, decreased the stimulation observed on addition of the latter components alone. In experiment 4, the juice, instead of being dialyzed, was a one-week-old preparation. A slight stimulation was observed in the activity of the juice on the addition of calcium pantothenate, and since pantothenic acid is fairly unstable (Rosenberg, 1943), these results may be a clue as to its necessity in this dissimilation.

In view of all the qualifications attending these experiments with pantothenic acid and biotin, it seems that no definite conclusions can be drawn as to the necessity of these components in the dissimilation of pyruvate; however, it is felt that they are involved in some way in this dissimilation.

Table 16. Summary of calcium pantothenate and biotin effects on dissimilation of pyruvate by E. coli enzyme preparation.

	Experiment No.			
	1	2	3	4
	(μl. CO_2 evolved)			
Dialyzed juice	71	0	16	41
DIAL. juice + $PO_4^=$	162	82	25	82
DIAL. juice + $PO_4^=$ + biotin	306	153	83	97
DIAL. juice + $PO_4^=$ + Ca-pantothenate	633	118	49	120
DIAL. juice + PO_4 + cocarb. + Mn			409	
Time	4½ hrs.	20 mins.	2½ hrs.	1 hr.

NOTE: Concentration of substances per cup: juice, 1.0 ml.; phosphate (pH 6.88), 0.02 M; biotin, 2μg.; Ca-pantothenate, 35μg.; cocarboxylase, 15μg.; Mn, 0.05 M; pyruvate, 0.045 M; $NaHCO_3$, 0.045 M. Total volume of each cup, 2.3 ml.; atmos., 10% CO_2 in H_2; temp., 30.4° C. Experiments 1 and 2: juice dialyzed 25 minutes. Exp. 3: juice dialyzed 85 minutes. Exp. 4: juice undialyzed, 7 days old.

Summary and conclusions

An active, cell-free extract has been obtained from E. coli, which attacks pyruvate anaerobically, producing acetic, formic, lactic, and succinic acids, and CO_2.

The enzyme system is active within a pH range of 6.2 to 7.0 and can be dried in vacuo without any immediate loss of activity. The dried preparation slowly deteriorates with age.

The preparation contains very strong formic dehydrogenase and hydrogenase activity. Formic dehydrogenase does not seem

to be associated with solid particles. Both enzyme systems are quite stable and can be converted to a dry powder, in which form they retain their activity for some time.

Inorganic phosphate, Mn^{--} and cocarboxylase were shown to be components of the enzyme system in the anaerobic dissimilation of pyruvate. The optimal concentration of phosphate was 0.018 M. At higher concentrations no further increase in the rate of dissimilation was observed.

Comparisons were made between the bacterial enzyme systems responsible for the anaerobic dissimilation and the aerobic oxidation of pyruvate. The two systems were found to be quite similar.

Dialysis for 1-1/2 to 2 hours inactivates the system. Addition of various compounds, alone or in combination, did not restore the activity, whereas the addition of boiled yeast juice had some effect.

Evidence is presented showing possible effects of pantothenic acid and biotin on dissimilation of pyruvate by this enzyme preparation.

CO_2-Fixation and Succinic Acid Formation

Since the discovery in 1935 that carbon dioxide is assimilated by heterotrophic bacteria, this concept has slowly been extended to other tissues and forms of life, so that at present

heterotrophic CO_2-utilization has almost reached the status of a general biological reaction. Availability of carbon isotopes provided a valuable tool in tracing the course of the fixed carbon dioxide, but it soon became evident that, in order to follow this reaction more accurately, enzyme preparations would have to be obtained which would be able to actively fix carbon dioxide. Attempts to further elucidate the mechanism of the fixation through the use of enzyme systems have only recently been inaugurated. Krampitz and Werkman (1941), Werkman et al. (1942), and Krampitz et al. (1943), using an acetone preparation of Micrococcus lysodeikticus, presented the first direct evidence that oxalacetic acid is an intermediate of the fixation reaction. During the decarboxylation of oxalacetate, there was an exchange between the $C^{13}O_2$ of the medium and the carboxyl group adjacent to the methylene carbon atom of the oxalacetic acid. Thus they demonstrated the reversibility of the fixation reaction:

$$CO_2 + CH_3.CO.COOH \rightleftharpoons HOOC.CH_2.CO.COOH$$

These investigators also demonstrated that magnesium ions are necessary for exchange to take place.

In a preliminary communication, Evans et al. (1942), working with phosphate buffer extracts of acetone-dried pigeon liver, obtained fixation of $C^{11}O_2$, but did not trace the radioactive carbon. Dialysis resulted in some loss of activity of the preparation. In a subsequent communication (Evans et al., 1943), it was demonstrated that when the enzyme was incubated

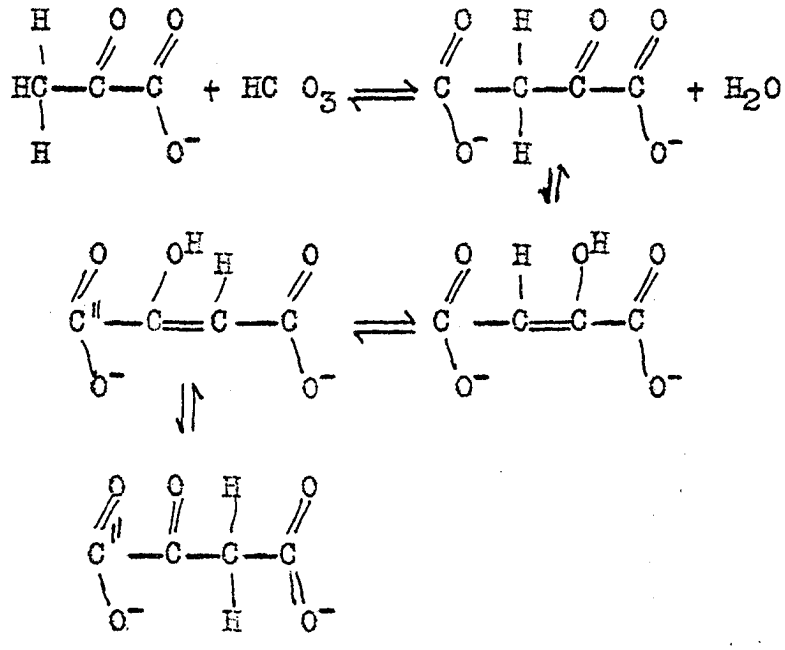
with pyruvate and $NaHC^{11}O_3$, no chemical change could be detected in the reaction mixture, but a small amount of C^{11} was fixed in the pyruvate. When fumarate was added to the pyruvate, enzyme, and $NaHC^{11}O_3$, a larger amount of $C^{11}O_2$ was fixed. Fifteen per cent of the fixed carbon dioxide was found in the pyruvate, and the remaining 85 per cent in the filtrate. In view of the lack of other soluble intermediates, those investigators claimed the radioactive carbon must exist as lactate, malate, and fumarate, although it was not traced to these compounds. The following chemical changes were postulated as occurring in the presence of fumarate and malate:

$$\text{fumarate} + H_2O \rightleftharpoons \text{malate}$$

$$\text{malate} + \text{pyruvate} \rightleftharpoons \text{oxalacetate} + \text{lactate}$$

$$\text{oxalacetate} + H_2O \rightleftharpoons \text{pyruvate} + \text{bicarbonate}$$

Thus there was no net change in the amount of pyruvate present, before and after the experiment. Carbon dioxide was produced, and the amount of lactate formed was equal to the amount of fumarate (+ malate) missing. The preparation contained an enzyme catalyzing the decarboxylation of oxalacetate, and the authors explained the presence of radioactive carbon in the pyruvate by postulating the occurrence of the following series of reactions:



Thus by postulating a dynamic shift of the hydroxyl group of enoloxalacetic acid (Meyerhoff, 1942), $C^{11}OOH.\underset{\underset{O}{\|}}{C}.CH_2.COOH$ is formed, which, on decarboxylation of the carboxyl group adjacent to the methylene group, would result in pyruvate containing radioactive carbon. The position of the radioactive carbon was not traced to any one definite compound other than to pyruvate, which contained only 15 per cent of the fixed C^{11}, and no net uptake of CO_2 was demonstrated.

Other than these two preparations, (1) the acetone preparation of Micrococcus lysodeikticus and (2) the extracts of acetone-dried pigeon liver, no other enzyme system has been described which can bring about fixation of carbon dioxide.

This phase of the investigation presents quantitative data for the fixation reaction brought about by the enzyme preparation of E. coli during the anaerobic dissimilation of pyruvate. An actual net uptake of carbon dioxide was observed, with pyruvate as substrate, and with CO_2 containing an excess of heavy carbon present; fixed $C^{13}O_2$ was located in the carboxyl groups of succinic, formic, and lactic acids. Another mechanism for succinic acid formation besides that of CO_2-utilization is also demonstrated with this enzyme preparation, in which the condensation of $CH_3.C^{13}OOH$ is established. The mechanisms for the formation of succinic and formic acids are discussed.

Demonstration of CO_2-utilization

Pyruvate is attacked anaerobically by the enzyme preparation, and carbon dioxide is evolved in manometric experiments with bicarbonate present. Occasionally, however, the carbon dioxide evolution fell far short of the usual values, although identical amounts of pyruvate had been fermented. Table 17 shows the carbon dioxide evolution in seven identical Warburg vessels. Despite the difference in the carbon dioxide evolved between cups one and seven, it was found on analysis that identical amounts of pyruvate were utilized in the two cups. Another peculiar, and perhaps important, observation was made. The CO_2 evolution in cups 2 and 6 ceased after approximately 40 minutes, and a direct gaseous uptake was observed, as

indicated. Such results seemed to point to the utilization of carbon dioxide, and, in view of the possibility that these were not the optimal conditions for the fixation of CO_2, experiments were carried out under different gases (Table 18). The CO_2 evolved during the course of the fermentation and the residual CO_2 evolved on acidification after completion of the fermentation equals the total CO_2. Column 4 gives the CO_2 obtained by simultaneously tipping the pyruvate and sulfuric acid into the Warburg vessel containing the enzyme preparation and the bicarbonate. The difference between columns 3 and 4 represents the carbon dioxide produced, or utilized in the fermentation. It is seen that under a nitrogen atmosphere 107 μl. of carbon dioxide were produced during the course of the fermentation. Under an atmosphere of hydrogen, much less carbon dioxide is produced, and in one instance, there was a slight loss. However, under atmospheres of 5 per cent and 10 per cent carbon dioxide in hydrogen, carbon dioxide was consistently unaccounted for in every case. The amount of CO_2 utilized was smaller under an atmosphere of 10 per cent CO_2 in N_2 whereas increasing the percentage of carbon dioxide to 50 and 100 per cent resulted in a net production of CO_2. Variation of the concentration of either the juice, the bicarbonate or the pyruvate invariably brought about a much smaller utilization of CO_2 and frequently caused a net production of CO_2.

The contents of the five experimental cups under an atmosphere of 10 per cent carbon dioxide in hydrogen were

Table 17. Variation in evolution of CO_2 in dissimulation of pyruvate by enzyme preparation.

Cup No.	CO_2 evolved (μl.)	Gas taken up (μl.)	Pyruvate utilized (%)
1	1771	0	98.8
2	929	266	
3	1495	0	
4	1615	0	
5	1349	0	
6	801	322	98.8
7	1142	0	

NOTE: Each cup contained pyruvate, 0.06 M; $NaHCO_3$, 0.05 M; juice, 1.6 ml. Total volume, 2.3 ml. Atmosphere, CO_2. Time, 2 hours. Temperature, 30.4° C.

carefully rinsed out and the precipitated proteins filtered off. Volatile acids were removed by steam distillation and the residue was extracted with ether over night. The ether extract was tested for the presence of succinic acid with a succinic dehydrogenase preparation obtained from beef heart. Succinic acid found amounted to 760μl. (on the basis that two moles of succinate take up one mole of O_2, and that 1 mM. succinate is equivalent to 22,400μl.). The CO_2 unaccounted for also was 760μl. Thus carbon dioxide was fixed and succinic acid formed in equimolar amounts.

Table 18. Effect of different gases on fixation of CO_2 by E. coli juice.

Atmosphere	CO_2 evolved (l.)				
	1	2	3	4	
	During fermentation	On acidifying after fermentation	Total (1-2)	On acidifying before fermentation	CO_2
N_2	332	819	1151	1044	+107
H_2	335	841	1176	1171	-5
	363	862	1225	1245	+20
5% CO_2 in H_2	308	950	1258	1412	-154
	318	940	1258	1412	-154
10% CO_2 in H_2	420	1158	1578	1670	-92
	426	1118	1544	1670	-126
	381	1187	1568	1720	-152 -760
	340	1202	1542	1720	-178
	370	1138	1508	1720	-212
10% CO_2 in N_2	477	1105	1582	1634	-52
50% CO_2 in H_2	459	1624	2083	1969	+114
	463	1610	2073	1969	+104
100% CO_2	667	2296	2963	2888	+75
	610	2299	2909	2888	+21

NOTE: Each cup contained 0.8 ml. juice, 0.022 M pyruvate, and 0.04 M $NaHCO_3$. Total volume 2.3 ml. Time, 3 hrs. Temp., 30.4° C.

Accordingly, with this indication that carbon dioxide is fixed and succinic acid is formed in equivalent amounts, experiments were carried out on a larger scale with excess C^{13} in the bicarbonate of the medium. Pyruvate and $NaHC^{13}O_3$ were placed in a 300 ml. Erlenmeyer flask which was connected to a condenser, and then to a bead tower containing 15 ml. of 1.5 N CO_2-free NaOH. The whole system was put under a slight vacuum and the air above replaced with CO_2-free H_2. Acid phosphate was added to liberate some $C^{13}O_2$ and to give an atmosphere of $C^{13}O_2$ in H_2. The enzyme was then added. After completion of the fermentation, the proteins were precipitated and residual CO_2 liberated by the addition of 10 ml. of 20% metaphosphoric acid. The solution was refluxed and aerated to complete the removal of CO_2. The resulting CO_2-free mixture was filtered and from the filtrate the fermentation products were isolated and analyzed for the presence of excess C^{13}. Results are shown in Table 19. The concentration is expressed as per cent C^{13} in excess of the normal complement of C^{13}, i.e., the percentage in excess of 1.09. The bicarbonate is the only source of carbon containing C^{13} in excess of 1.09, and the variation in the mass spectrometer analysis of C^{13} is ±0.02. The excess C^{13} values of the succinic, formic, and lactic acids are significant and indicate fixation of CO_2.

Mechanism of succinic acid formation

The succinic acid isolated from the fermentation was converted to a mixture of fumarate and malate by means of a

succinic dehydrogenase preparation obtained from beef heart. The malate was then oxidized with $KMnO_4$ to acetaldehyde plus 2 CO_2. The two carbon dioxide molecules originate from the two carboxyl groups, and acetaldehyde from the methylene groups of the original succinic acid (Wood et al., 1941). The acetaldehyde was then oxidized to CO_2 with potassium persulfate (Osburn and Werkman, 1932) and the C^{13} content of the carboxyl and of the methylene carbons determined. The C^{13} content of the methylene carbons of succinic acid was found to be normal (1.09%), whereas the figures for the carboxyl groups showed that a significant amount of excess C^{13} was present. The results conclusively indicate that there has been fixation of carbon dioxide by the enzyme preparation of Escherichia coli with pyruvate as substrate, and that the fixed carbon is located in the carboxyl groups of the succinic acid formed. The values for the carboxyl carbons are not high enough to indicate that all of the succinate has been formed by a fixation of CO_2. If the fixed $C^{13}O_2$ is present in only one of the carboxyl groups of the succinic acid, according to the reactions

$$C^{13}O_2 + HCH_2.CO.COOH \longrightarrow C^{13}OOH.CH_2.CO.COOH$$

$$C^{13}OOH.CH_2.CO.COOH + 4H \longrightarrow C^{13}OOH.CH_2.CH_2.COOH + H_2O$$

(Wood et al., 1941), the excess C^{13} in the single carboxyl group containing fixed C^{13} will be 0.84 and 1.30, i.e., twice as large as the value for the two carboxyl groups. These values are much lower than the excess C^{13} in the bicarbonate (4.68 and

Table 19. Per cent C^{13} in products of pyruvate and $NaHC^{13}O_3$ dissimilation by E. coli enzyme preparation.

	Fermentation No. 1		Fermentation No. 2	
	mM	% excess C^{13}	mM	% excess C^{13}
Original $NaHCO_3$	3.966	5.10	5.295	5.10
Residual $NaHCO_3$	4.178	4.68	5.465	4.17
Pyruvate fermented	2.85		2.67	
Products:				
CO_2	0.212		0.17	
Formic	2.30	0.16	1.85	0.08
Acetic	2.30	0.00	2.03	0.00
Lactic (COOH)	0.07	0.61	0.10	0.27
Succinic	0.24		0.383	
Succinic $(COOH)_2$		0.42		0.65
Succinic $(CH_2)_2$		0.00		0.00

Fermentation No. 1 contained: 30 ml. enzyme preparation; $NaHC^{13}O_2$, 0.05 M; pyruvate, 0.05 M; phosphate, 7 ml. (0.1 M, pH 6.2). Total volume, 75 ml. Atmosphere $C^{13}O_2$ in H_2. Time, 4 hours. Temp., 30.4° C.

Carbon recovery 96%; O/R index 1.03; excess C^{13} recovered 100%.

Fermentation No. 2 contained: 38 ml. enzyme preparation; $NaHC^{13}O_3$, 0.07 M; pyruvate, 0.04 M; phosphate, 10 ml. (0.1 M, pH 6.2). Total volume, 75 ml. Atmosphere, $C^{13}O_2$ in H_2. Time, 4 hours. Temp., 30.4° C.

Carbon recovery 99%; O/R index 0.97; excess C^{13} recovered 87%.

4.17) and it is evident the carboxyl was not derived solely from this source. Assuming that the $C^{13}O_2$ is in equilibrium with the $C^{12}O_2$ in the medium and at the enzyme surfaces,

Table 20. C^{13} in succinate formed in quantities equimolar to carbon dioxide utilized.

CO_2 missing (µl.)	Succinate formed (µl.)	Excess C^{13} in total succinate (%)	Excess C^{13} in carboxyl groups, calculated (%)
1311	1368	0.21	0.42

NOTE: Each cup contained: juice, 0.8 ml.; pyruvate, 0.024 M; $NaHC^{13}O_3$, 0.046 M; phosphate, 0.2 ml. (0.1 M, pH 6.2); H_2SO_4 (1:1), 0.3 ml. (side arm). Total volume, 2.3 ml. Atmosphere, $C^{13}O_2$ in H_2. Time, 3 hours.

then, if all the succinate were formed by a fixation of CO_2, the C^{13} content in the carboxyl group of the succinate should be at least as large as the C^{13} content in the bicarbonate at the end of the fermentation. Since this was not so, either the isotopes are not at equilibrium at the enzyme surfaces, or there is another mechanism of succinic acid formation. It will be recalled that in the manometric experiments (Table 18) the succinate was formed in amounts equimolar to the CO_2 utilized, whereas in the larger experiments (Table 19) there was a small net production of CO_2 during the course of the fermentation. In order to ascertain whether succinate contains a higher complement of fixed C^{13} when it is formed in quantities equimolar to the CO_2 utilized, small manometric experiments were set up with $NaHC^{13}O_3$, and the C^{13} of the isolated succinic acid was determined (Table 20). The quantity of 1311 µl. CO_2 was utilized in eleven Warburg cups, and 1368 µl. succinate was

formed. However, the excess C^{13} content of the carboxyls of the succinate formed (0.83%) was not higher than that obtained in large-scale experiments (0.84 and 1.30%), where no net loss of CO_2 occurred. Here again, though the amount of succinate formed correspond to the CO_2 utilized, the C^{13} content of the resulting succinate indicated that there was another mechanism for succinic acid formation besides that of CO_2 utilization.

Acetic acid condensation

Thunberg (1920) and Wieland (1922) suggested the formation of succinic acid by a dehydrogenation of two moles of acetic acid. Since then, numerous investigators have presented evidence for this mechanism of succinic acid formation by yeasts (Wieland and Sonderhoff, 1932), molds (Butkevich and Fedorov, 1929, 1930), bacteria (Wood and Werkman, 1936b), and animal tissue (Weil-Malherbe, 1937). Sonderhoff and Thomas (1937) investigated the metabolism of yeast using trideuteroacetic acid and found deuterium in the succinate formed. The amount of deuterium in the succinate was less than that in the acetate. Nevertheless, their experiments clearly showed that succinate was derived from acetate, although the mechanism is probably more complex than simple dehydrogenation. Kleinzeller (1941), reported that yeast did not form succinate from acetate under the conditions of his experiments, and Foster et al. (1941) state that "the evidence for a synthesis of succinate through two acetates (Thunberg-Wieland condensation) has never been conclusive."

Confirmation of Sonderhoff and Wieland's work was necessary and was presented by Slade and Werkman (1943). These authors, working with Aerobacter, and using acetic acid containing C^{13}, obtained definite evidence for the formation of succinic acid by the condensation of two C_2 molecules, probably those of acetic acid.

On the basis that acetic acid condensation might be a mechanism of succinate formation with the enzyme preparation of Escherichia coli, a large-scale experiment similar to those described in Table 19 was carried out with pyruvate and $CH_3.C^{13}OOH$ as substrates. Table 21 shows the C^{13} content of the products of this dissimilation by the E. coli enzyme preparation. The only source of excess C^{13} was the added acetate. Succinic acid was the only compound other than acetic acid which was found to contain excess C^{13}. The heavy carbon of the succinic acid was located exclusively in the carboxyl groups. Since the residual CO_2 of the fermentation contained no excess C^{13}, it is evident that the heavy-carbon succinate was not formed by the fixation reaction. Therefore, this experiment conclusively shows that, besides the fixation reaction for succinate formation, the preparation has a second mechanism, the condensation of acetic acid (or its derivative) with a two- or three-carbon molecule (pyruvic acid or its derivative). The over-all reaction may be represented as follows:

$$C^{13}OOHCH_3 + CH_3.C^{13}OOH \longrightarrow C^{13}OOH.CH_2.CH_2.C^{13}OOH$$

Table 21. Per cent C^{13} in products of $CH_3C^{13}OOH$ and pyruvate dissimilation by E. coli enzyme preparation.

	mM.	Excess C^{13} (%)
$CH_3C^{13}OOH$ added	2.06	1.30
Pyruvate fermented	2.98	
Products:		
CO_2	0.30	0.01
Formic	2.14	0.02
Acetic	4.27	0.65
Lactic	0.07	0.02
Lactic COOH		0.02
Succinic	0.392	0.13
Succinic $(COOH)_2$		0.26
Succinic $(CH_2)_2$		0.02

NOTE: Fermentation contained: pyruvate, 0.038 M; $CH_3C^{13}OOH$, 0.024 M; $NaHCO_3$, 0.064 M; phosphate, 2 ml. (0.5 M, pH 6.2); enzyme preparation, 30 ml. Total volume, 85 ml. Atmosphere, CO_2 in H_2. Time, 4 hours. Temp., 30° C.

Carbon recovered, 97%
O/R index, 1.05
Per cent excess C^{13} recovered, 107%

The present evidence does not establish that it is specifically two molecules of acetic acid which react, but it does establish that acetic acid can be converted to succinic acid by condensation of two molecules of acetic acid or by a reaction

involving acetic acid. Slade and Werkman (1943) have also demonstrated with Aerobacter the reverse reaction, i.e., the cleavage of succinate to acetate. This evidence supports the proposal of the formation of succinate by a C_2 plus C_2 addition, rather than by a 2-carbon plus 3-carbon addition. The enzyme system reported here apparently does not possess this cleavage mechanism, since, in the presence of $NaHC^{13}O_3$ (Table 19), the succinic acid contained an excess of C^{13}, whereas the C^{13} content of the acetic acid was normal.

Since two mechanisms for succinic acid formation have been demonstrated with this preparation, the question arises as to the percentage of the succinate formed by each mechanism. The quantitative isotopic data can be used to give some information, although there are limitations that have to be considered. The calculations are not exact because of uncertainty as to mechanisms of the reactions, assumption of equilibria between the C^{13} and C^{12} compounds at the enzyme surfaces and the difficulty in determining the concentration of the C^{13} compounds during the course of the fermentation. Minimal values can be calculated, but the calculation of maximum or exact values involves assumptions.

The acetate added at the beginning of the experiment (Table 21) contained 1.30 per cent excess C^{13}. If the C^{13} content of these acetic acid molecules is not diluted by acetic acid arising from pyruvate, and if condensation takes place between two acetic acid molecules, each molecule of succinate

formed will contain 1.30 per cent excess C^{13}, i.e., the same percentage as the added $CH_3.C^{13}OOH$. The experimental value for the succinate is 0.13% excess C^{13}. If x = the percent of succinate formed by condensation, then, if X moles of succinate containing 1.30% excess C^{13} are diluted with ordinary succinate to 100 moles, the resulting succinate will contain 0.13% excess C^{13}.(1.30 x X = 100 x 0.13). The value for the succinate formed by condensation of $CH_3.C^{13}OOH$ containing 1.30% excess C^{13} is therefore 10%. This is the lowest possible value for acetate condensation which could give the experimental value of 0.13%.

If the succinate arose by condensation of one molecule of added acetate with one molecule of pyruvate, the excess C^{13} content of the resulting succinate will be one half the acetate, or 0.65%. The succinate formed by condensation will be 20% in this case (0.65 x X = 100 x 0.13).

The above calculations are not correct for it is apparent that acetate arising from the pyruvate will also condense. Thus, the acetate which enters the condensation reaction will have a C^{13} concentration less than that of the original added acetate, but somewhat greater than that of the final acetate. The per cent succinate formed by condensation as calculated on the basis of the final acetate is probably a maximum value. If both portions of the succinate come from the final acetate, the value is 20% (0.65 x X = 100 x 0.13). If the succinate is formed by acetate and pyruvate condensation, the calculation

on the basis of the C^{13} content of the final acetate gives 40% (0.325 x X = 100 x 0.13).

On the same basis, and assuming the CO_2 is fixed in only one carboxyl group (cf. Krampitz et al., 1943), we can calculate the per cent of succinate arising from CO_2-fixation to be between 16.5 (5.10 x X = 100 x 0.84) and 18% (fermentation No. 1, Table 19) and between 25 and 31% (fermentation No. 2, Table 19).

Even if the maximal values are taken for the per cent of succinic acid formed by CO_2 utilization and acetic condensation, only 71% of the succinate mechanism is accounted for. The results were not from the same fermentation and there may have been some variation. We have no assurance, however, that the assumptions on which these calculations are based are entirely correct. For example, the $C^{12}O_2$ produced at the enzyme surfaces may be more closely bound to the enzyme and therefore may be preferentially fixed, even though both may be in equilibrium in the medium. There is no direct evidence that the added $CH_3.C^{13}OOH$ comes to equilibrium with the acetate formed from pyruvate, or with an intermediate two-carbon molecule which is actually condensed to form succinic acid. Finally there is the possibility that there may be at least one more mechanism of succinic acid formation by this enzyme preparation, besides the two indicated above.

The principal point to be made is that there are at least two mechanisms of succinate formation which are of quantitative

significance. Therefore, further investigation is necessary in cases where only one mechanism for succinate formation is postulated (Krebs and Eggleston, 1941).

Mechanism of formic acid formation

At present there are two concepts for the formation of formic acid from pyruvate: (1) the hydroclastic split, in which pyruvic acid breaks down to acetic and formic acids, $CH_3.CO.COOH + H_2O = CH_3.COOH + HCOOH$, and (2) reduction of CO_2 by either gaseous hydrogen or an organic hydrogen donator. Neuberg (1914) showed the conversion of pyruvate to acetic and formic acids. He pictured the over-all reaction as a hydroclastic split of pyruvate to acetic and formic acids but also proposed a mechanism for the formation of formic acid by the reduction of carbonic acid (split off from pyruvate) by the nascent hydrogen arising from the water in the medium. Tikka (1935) proposed the transformation of pyruvic acid by E. coli entirely without decarboxylation through a hydroclastic split into equimolar amounts of acetic and formic acids. Krebs (1937) proposed that formate was not necessarily formed from the carboxyl group of pyruvic acid but could arise from the CO_2 and water of the medium, according to the equation

$$CH_3.CO.COOH + H_2O + CO_2 = CH_3.COOH + CO_2 + HCOOH$$

His point was that this mechanism was possible, and the hydroclastic split not definitely proven. Werkman and Wood (1942) stated that it was generally accepted that formic acid originates from pyruvic acid by a hydroclastic split. However,

these authors pointed out the possibility that CO_2 could be reduced by an organic hydrogen donator to form formic acid.

Woods (1936) has shown that the reduction of CO_2 to HCOOH with H_2 occurs with E. coli and presumably with other bacteria that contain hydrogenlyase. The E. coli enzyme preparation used in these experiments was obtained from cells which were grown under strong aeration, with no formate present, and consequently did not contain any hydrogenlyase when tested (Yudkin, 1932). In the presence of $C^{13}O_2$, the absence of an excess of heavy carbon in the formic acid formed from pyruvate dissimilation would indicate that formic acid was not formed by reduction of CO_2 as such. The results of such experiments are presented in Table 19. It is evident that the per cent of heavy carbon present in the formic acid in both fermentations is significant, but only slightly above the normal. Bearing in mind the fact that, with hydrogenlyase present, the C^{13} content of the formic acid formed is equal to the C^{13} content of the residual bicarbonate (Slade et al., 1942), it is evident that when no hydrogenlyase is present, as in this instance, very little of the formic acid is formed by a reduction of CO_2 from the medium. Calculating the per cent of formic acid arising from a reduction of CO_2, according to the C^{13} content of the formic acid formed and of the residual and original bicarbonate (Table 19), we find that in fermentation No. 1 from 3.2 to 3.5% of formate originated in this way, and

in fermentation No. 2, 1.2 to 1.8% of the formate was formed by a reduction of CO_2 from the medium. A slight residual hydrogenlyase activity might account for the small excess amount of C^{13} present in the formic acid. On the other hand, the C^{12} formed from pyruvate may be more closely bound to the enzyme surfaces and thus may be preferentially reduced. In this case, however, the formation of formic acid would not be the result of the reduction of CO_2 from the medium.

It appears that the reduction of CO_2 is probably not the mechanism by which formic acid arises in this case. However, the hydroclastic split, as pictured, is probably incorrect also, since phosphate is known to be necessary for this reaction, for the oxidative breakdown of pyruvate and for the conversion of pyruvate into acetate, CO_2, and molecular hydrogen.

Lipmann (1940,1941) has demonstrated the occurrence of acetyl phosphate during the oxidation of pyruvate by extracts of dried Lactobacillus cells. More recently, Utter and Werkman (1943) have shown the occurrence of this phosphorylated product during the anaerobic dissimilation of pyruvate by a cell-free enzyme preparation of E. coli. Therefore, it would probably be more correct to picture the formation of acetic and formic acids from pyruvic acid as a phosphoroclastic reaction instead of a hydroclastic reaction.

Lactic acid formation

In the dissimilation of pyruvic acid, very small amounts of lactic acid were formed. However, a significant amount of

heavy carbon was fixed in the lactic carboxyl group in the presence of $NaHC^{13}O_3$. These experiments confirm some of the previous work done with whole cells, where, concurrent with CO_2 utilization in succinic acid, heavy carbon was also found in the carboxyl group of the lactic acid produced by many organisms (Wood *et al.*, 1942, and Slade *et al.*, 1942). These results seem to suggest that a C-4 dicarboxylic acid, formed by a C-3 and C-1 addition, may be a precursor to the formation of lactic acid. However, from the results in Table 21, page 90, it can be seen that succinic acid itself is probably not an intermediate in the formation of lactic acid. The succinic acid molecule contained an excess of heavy carbon (derived from the $CH_3C^{13}OOH$), whereas the C^{13} content of the lactic carboxyl group was practically normal.

In view of the small amounts of lactic acid formed by this system, it would be inadvisable to try to formulate a mechanism for its formation, on the basis of the data in this paper. The mechanism of this reaction would probably be studied to greater advantage by using an organism or an enzyme system producing much larger amounts of lactic acid.

Summary and conclusions

The dissimilation of pyruvate has been investigated using an active, cell-free enzyme preparation obtained from *E. coli*. The products are succinate, lactate, acetate, formate, and CO_2. CO_2 was utilized and, by the use of tracer experiments with the C^{13} isotope, fixed $C^{13}O_2$ was located in the carboxyl group of

the succinic acid formed.

CO_2-fixation is not the only mechanism of succinic acid formation by this enzyme system. On addition of $CH_3C^{13}OOH$, succinic acid was isolated, containing excess C^{13} exclusively in the carboxyl groups. Therefore, condensation of acetic acid, or its derivative, with a 2-carbon or 3-carbon molecule is another mechanism for the formation of succinic acid.

It is shown that in the absence of hydrogenlyase the reduction of CO_2 from the medium is not the mechanism of formic acid formation.

Thus, with the knowledge that the bacterial preparation contained the enzyme or enzymes which actively fixed carbon dioxide with pyruvate as substrate, the next phase of this investigation resolved itself into attempts to determine some of the properties of the system concerned with the enzymic decarboxylation of oxalacetate and the demonstration of the carboxylation of pyruvate.

Enzymic Decarboxylation of Oxalacetate and Carboxylation of Pyruvate

Oxalacetate is an important intermediary in cellular metabolism and its decarboxylation and reduction by many microorganisms and animal tissues have been reported by a number of investigators. The first bacterial enzyme preparation to be described, which brought about a rapid decarboxylation of oxalacetate was obtained from Micrococcus lysodeikticus by

Krampitz and Werkman (1941). This enzyme was heat labile, in contrast to the heat-stable component of mammalian tissue described by Breusch (1939).

The formation of oxalacetate from malate and fumarate has been determined by a number of investigators. Banga (1936), using hydrazine as a fixative, demonstrated the formation of oxalacetate from fumarate, with washed muscle. Stare (1936) also showed the oxidative formation of oxalacetate from fumarate by liver and kidney tissues. Malic dehydrogenase from pig heart was shown to form oxalacetate from l-malate, in the presence of keto-fixatives (Green, 1936b). Among bacteria, the formation of oxalacetate from fumarate was demonstrated by Krampitz et al. (1943). However, no quantitative data were given. Attempts of these workers to demonstrate the formation of oxalacetate via a direct carboxylation of pyruvate were unsuccessful. However, employing a bacterial acetone preparation and the heavy carbon isotope, they did demonstrate that during the decarboxylation of oxalacetate to pyruvate and CO_2, some carboxylation occurred. This was the first direct evidence that oxalacetic acid (or its derivative) was a component of the fixation reaction and that this reaction was reversible:

$$CO_2 + CH_3.COCOOH \rightleftharpoons COOH.CH_2.CO.COOH$$

In the present investigation, the enzyme preparation obtained from E. coli has been used to confirm and extend the work reported by these investigators. The enzyme system

exhibits strong decarboxylating activity on oxalacetate, and in addition, can reduce oxalacetate and fumarate with molecular hydrogen. Quantitative data are presented for the formation of oxalacetate (or a compound very closely related to it) from the 4-carbon dicarboxylic acids and directly from pyruvate and CO_2 *via* the Wood-Werkman fixation reaction.

Activity on fumarate and oxalacetate

Strong activity was exhibited with fumarate and oxalacetate as acceptors of gaseous hydrogen (Table 22). This is of interest in view of the postulated occurrence of these two compounds in the formation of succinic acid from pyruvate. The reduction of oxalacetate is much slower than that of fumarate. However, oxalacetate is rapidly decarboxylated, a fact which suggests a similarity between this system and the acetone preparations of *Micrococcus lysodeikticus* (Krampitz and Werkman, 1941), and of pigeon liver (Evans *et al.*, 1943). Krampitz and Werkman found that magnesium (or manganese) ions are necessary for the decarboxylation of oxalacetate, and Evans demonstrated that manganese ions functions in the corresponding enzyme system in pigeon liver. In addition, the former investigators found that cocarboxylase did not function in oxalacetate decarboxylation. This work has been repeated with a dialyzed *E. coli* enzyme preparation and substantially the same results were obtained. In addition it was demonstrated that inorganic phosphate is not necessary for the decarboxylation of oxalacetate.

Table 22. Activity of β-decarboxylase preparation on fumarate and on oxalacetate.

	CO_2 evolved (μl.)	H_2 uptake (μl.)
Fumarate	(-472) 0	-428
	(-490) 0	-557
Oxalacetate	+413	-96
	+443	-92
	+446	-106
	+412	-112
Oxalacetate (spontaneous)	+87	-4
	+84	

NOTE: Figures not corrected for spontaneous action of oxalacetate. Substrate concentration, 0.02 M. Enzyme, 0.8 ml. (In oxalacetate control, 0.8 ml. phosphate, 0.2 M; pH, 6.88, added instead of juice.) Total cup contents, 2.0 ml. Atmosphere, H_2. Time, 1 hr.

Properties of the enzyme system

It will be recalled that there was a sharp dilution effect of the juice in the anaerobic dissimilation of pyruvate (Table 6). This dilution effect was not observed during the decarboxylation of oxalacetate (Table 23). Optimal activity was obtained with 0.7 ml. of the enzyme preparation, but even when as little as 0.1 or 0.2 ml. of the juice was used, definite activity was obtained.

Krebs (1942) has found that amino compounds, including amino acids, proteins, aniline and multivalent ions also

Table 23. Effect of dilution of juice on decarboxylation of oxalacetate.

Juice (ml.)	CO_2 evolved (μl.)
0.8	1003
0.7	1110
0.6	799
0.5	700
0.4	640
0.3	568
0.2	432
0.1	234
--	0

NOTE: Each cup contained: juice in indicated amounts; oxalacetate, 0.045 M; phosphate buffer (pH, 6.88), 0.05 M; citric acid (side arm, 50%), 0.3 ml. Total volume, 2.0 ml. Time, 0.5 hr. Atmosphere, air. Temperature, 30.4° C. μl. CO_2 evolved = CO_2 evolved during 0.5 hr. plus CO_2 evolved on acidification. Figures corrected for spontaneous action of oxalacetate.

catalyze this β-decarboxylation. There is no doubt that Krampitz and Werkman (1941) demonstrated a heat-labile enzyme which decarboxylates oxalacetic acid, and the preparation described here is quite similar. The specific protein nature of the enzyme was demonstrated by the precipitation of the proteins responsible for the decarboxylation with 50% $(NH_4)_2SO_4$ (Table 24).

Table 24. Fractional precipitation of -decarboxylase.

$(NH_4)_2SO_4$ (%)	CO_2 evolved from oxalacetate (µl.)
60	301
50	236
45	19
40	29

NOTE: Each cup contained: juice, 1.0 ml.; oxalacetate, 0.025 M; phosphate buffer (pH 6.88), 0.05 M.; citric acid (in side arm, 50%), 0.3 ml. Total volume, 2.0 ml. Atmosphere, air. Time, 0.5 hr. Corrected for spontaneous decarboxylation of oxalacetate.

The protein fraction which was precipitated with 45% $(NH_4)_2SO_4$ had no decarboxylating activity on oxalacetate and did not increase the activity of the protein fraction precipitated by 50% $(NH_4)_2SO_4$, on addition to it. Therefore, though proteins and amino acids may catalyze the spontaneous decarboxylation of oxalacetate, there is a specific protein system which brings about a much more rapid decarboxylation. The activity of the enzyme seemed quite unaffected by the pH of the medium, since oxalacetate was decarboxylated within a pH range of 2 to 10. The enzyme appeared saturated when the concentration of oxalacetate was approximately 0.05 M (Table 25), compared to approximately 0.03 M pyruvate concentration necessary for

Table 25. Effect of substrate concentration on enzyme saturation in decarboxylation of oxalacetate.

Oxalacetate concentration (M)	CO_2 evolved (µl.)
0.01	206
0.03	300
0.04	313
0.05	384
0.06	375

NOTE: Each cup contained: juice, 0.3 ml.; oxalacetate in indicated concentrations; phosphate buffer (pH, 6.88), 0.05 M; citric acid (side arm, 50%), 0.3 ml. Total volume, 2.0 ml. Atmosphere, N_2. Time, 0.5 hr. µl. CO_2 includes CO_2 evolved during fermentation plus CO_2 evolved on addition of citric acid. Correction made for spontaneous decarboxylation of oxalacetate.

saturation during dissimilation of pyruvate with the same preparation (Table 7).

Formation of oxalacetate from succinate, fumarate, and malate

Quantitative data have been obtained on the formation of oxalacetate from succinate, fumarate, and malate, employing the cell-free enzyme preparation. The presence of oxalacetate was determined by its decarboxylation on addition of aniline-citrate (Edson, 1935). The action of the juice was stopped at the desired time by the addition of citric acid to the

center well of the Warburg cup, thus releasing any bound CO_2 which might be present. Oxalacetate is rapidly decarboxylated in the presence of aniline-citrate, and thus its presence can be determined quantitatively. Aniline-citrate also slowly decarboxylates acetoacetate, but since this compound does not occur under the conditions of the experiment, the citrate-aniline test, in this instance, is considered specific for oxalacetate. In all cases, controls were run. Aerobically, with approximately 2,688 μl. of fumarate or malate present as substrate about 75 μl. of oxalacetate were obtained at the end of one hour, with no fixatives or inhibitors present (Table 26). Under the same conditions, much less oxalacetate (approximately 15 μl.) is formed from succinate. The oxalacetate formed was completely destroyed by heating to 70-80° C for five minutes, thus exhibiting the labile property of ordinary oxalacetate. However, under an atmosphere of N_2 with no O_2 present, no oxalacetate is formed from fumarate. Therefore it seems that this conversion to oxalacetate is an oxidative reaction.

The reduction of oxalacetate and fumarate by this enzyme preparation, plus the formation of oxalacetate from succinate, fumarate, and malate, demonstrate the possible and reversible occurrence of these intermediates during the utilization of carbon dioxide by this enzyme preparation.

Table 26. Aerobic formation of oxalacetate from various substrates by E. coli enzyme preparation.

Substrate	O_2 taken up (µl.)	CO_2 evolved (µl.)	Oxalacetate formed (µl.)
Fumarate	-42	65	75
"	-43	63	75
"	-46	60	
"	-41	66	
Fumarate control	0	0	2
Malate	-25	70	
"	-28	67	76
"	-35	58	
Malate control	0	0	-5
Succinate	-42	24	15
"	-46	20	15
Succinate control	0	0	1

NOTE: Each cup contained: juice, 0.8 ml.; substrate (fumarate, 0.05 M; malate, 0.05 M; succinate, 0.045 M); citric acid (side arm, 50%), 0.3 ml. Total volume, 2.3 ml. Time, 1 hour. Temperature, 30.4° C. Atmosphere, air. Cups run in duplicate with NaOH in center well to determine O_2 uptake and CO_2 evolution. Aniline-citrate added to determine oxalacetate, 0.4 ml.

Carboxylation of pyruvate

During the decarboxylation of oxalacetate by the enzyme in the presence of $NaHC^{13}O_3$, an exchange reaction took place; i.e., an excess of heavy carbon was detected in the carboxyl group adjacent to the methylene carbon atom of the residual oxalacetate. This is an indirect demonstration of the occurrence of carboxylation, and is similar to the results obtained

by Krampitz et al. (1943). Since CO_2-fixation had already been demonstrated with this enzyme preparation with pyruvate as substrate, attempts were made to demonstrate direct carboxylation by detecting the formation of oxalacetate from pyruvate and CO_2.

Under optimal conditions for succinic acid formation and CO_2-fixation by the juice, no test was obtained for oxalacetate (Table 28). An atmosphere of 5% CO_2 in H_2 was used, since under these conditions the fixation of CO_2 and the formation of succinic acid were previously demonstrated (Table 18). The CO_2 evolved during the course of the fermentation plus the CO_2 evolved on acidifying the contents of the Warburg vessels after permitting the fermentation to proceed for a definite length of time, equals the total CO_2. Duplicate control cups were acidified by simultaneously tipping the pyruvate and sulfuric acid into the center well of the Warburg vessel containing the enzyme preparation and the bicarbonate. The difference between the controls and the total CO_2 evolved from a cup where the fermentation was allowed to proceed, represents the CO_2 produced, or utilized in the fermentation. Thus, it can be seen (Table 27), that after 30 minutes there was an excess of 104 μl. of CO_2 produced and there was no test for oxalacetate. After 45 minutes the CO_2 excess was reduced to +22 μl. At 60 minutes 47 μl. of CO_2 were missing, but no exalacetate was detected. After 75 minutes and 180 minutes, the amount of CO_2 fixed increased to 117 μl. and 187 μl., respectively,

Table 27. Absence of oxalacetate formation under optimal conditions for CO_2 utilization by E. coli enzyme preparation.

Time (mins.)	CO_2 fixed* (μl.)	Oxalacetate formed (μl.)
10	+71	0
30	+104	0
45	+22	0
60	-47	-1
75	-117	0
180	-187	0

*The plus sign (+) means excess CO_2; the minus sign (-), CO_2 missing or fixed.

NOTE: Cups contained: juice, 0.8 ml.; pyruvate, 0.026 M; $NaHCO_3$, 0.039 M; citric acid (in side arm, 50%), 0.3 ml. Total volume, 2.3 ml. Atmosphere, 5% CO_2 in H_2. Temperature, 30.4° C. Citrate aniline added afterwards, 0.4 ml.

but in no case was oxalacetate detected. Any oxalacetate which might have been formed would almost immediately be reduced, due to the presence of molecular hydrogen.

Under the same conditions, but substituting N_2 for H_2 to prevent the reduction of any oxalacetate that might be formed, a definite test for oxalacetate was obtained (Table 28) on the addition of aniline-citrate to the medium after all bound CO_2 had been liberated. The exact procedure is described in the section on methods. The contents of the control cups were exactly the same and were treated in the same way as those of the experimental cups except that the pyruvate in the side arm was not tipped into the center well containing the juice until after the citric acid had been tipped in from the other

Table 28. Formation of oxalacetate from pyruvate and CO_2.

Atmosphere	Time (minutes)	Oxalacetate formed (μl.)	Controls
5% CO_2 in N_2	25	22	-2
	60	24	0
	90	12	0
5% CO_2 in H_2	30	0	0
	90	0	0

NOTE: Juice 0.8 ml.; pyruvate (0.3 M), 0.2 ml.; $NaHCO_3$ (0.3 M), 0.3 ml; citric acid (in side arm, 1:1), 0.3 ml.; H_2O to total volume of 2.3 ml. Temperature, 30.4° C. Citrate-aniline added afterwards, 0.4 ml.

side arm. Thus, the contents were the same, and the enzyme did not act on the pyruvate. Again, using the same juice, under an atmosphere of 5% CO_2 in H_2, no test was obtained for oxalacetate.

In time-experiments conducted on the formation of oxalacetate from pyruvate and CO_2, the amounts formed were, generally, slightly higher when the enzyme was allowed to act for 45 or 50 minutes. Smaller amounts of oxalacetate were detected at 20 minutes, and decreasing amounts were observed after a 60-minute period of activity. The quantities of oxalacetate formed (expressed in microliters of gas) were small, but each experiment was carried out at least in triplicate and the results checked well. Controls for each experiment were always run at least in duplicate under the exact conditions of the experiment and at the same time.

Effect of concentration of enzyme and substrate on carboxylation

If the formation of oxalacetate from pyruvate and CO_2 is definitely an enzymic reaction and an equilibrium does exist as postulated,

$$CO_2 + CH_3.CO.COOH \rightleftharpoons COOH.CH_2.CO.COOH$$

then definite effects should be observed on varying the concentrations of the enzyme, pyruvate, and carbon dioxide. The effect of concentration of enzyme on formation of oxalacetate from pyruvate is shown in Table 29. Here again, a dilution effect due to the concentration of enzyme was noted, for on decreasing the amount of juice from 0.8 to 0.4 ml., the amount of oxalacetate formed decreased by two-thirds. Eight-tenths milliliter of juice was found to be optimal, since on the addition of 1.0 ml. of the juice and subsequent acidification in the Warburg cup, longer shaking was necessary because of the greater amount of precipitated proteins present, which tended to delay slightly the complete liberation of all the bound carbon dioxide.

The effect of concentration of pyruvate on the formation of oxalacetate is shown in Table 30. It is evident that increasing concentrations of pyruvate results in quite small but definite increases of oxalacetate. Carbon dioxide was also shown to be necessary for the reaction (Table 31). During the anaerobic dissimilation of pyruvate by this enzyme preparation, a small amount of CO_2 is produced, and this amount

Table 29. Effect of concentration of enzyme on formation of oxalacetate from pyruvate.

Enzyme (ml.)	Oxalacetate formed (μl.)		
		Average	Controls
0.4	8		0
	7		0
	3	6	
0.6	11		0
	14	12	0
	11		
0.8	21		0
	18		0
	18	19	
1.0	14		0
	27		0
	12		
	25	20	

NOTE: Cups contained: juice in indicated amounts; pyruvate (0.3 M), 0.4 ml.; citric acid (1:1), 0.3 ml. (in side arm); H_2O to total volume of 2.0 ml. Time, 50 minutes. Atmosphere, 10% CO_2 in N_2. Temperature 30.4° C.

is sufficient to cause the formation of some oxalacetate (Experiment No. 2, Table 31). However, when NaOH is added to the alkali well of the Warburg vessel, during the dissimilation of pyruvate, the oxalacetate formed is decreased to zero in some cases. (The NaOH was removed before addition of citrate-aniline to the cup.) Further increase in the CO_2-concentration in the system by the addition of $NaHCO_3$, previously saturated with CO_2, resulted in an increase in the amount of oxalacetate formed, the optimal concentration of bicarbonate for formation of oxalacetate being 0.03 M (Table 31, Exp. 4).

Table 30. Effect of concentration of pyruvate on formation of oxalacetate.

Pyruvate concentration (M)	Oxalacetate formed (μl.)		
		Average	Controls
0.09	18		
	19		
	25	21	+1.5
0.070	17		
	24		-1.5
	21	21	+1.5
0.053	14		
	14		+1.5
	14	14	+4
0.035	11		
	10	10.5	0
0.018	0		
	0	0	0

NOTE: Each cup contained: juice, 0.8 ml; pyruvate in indicated concentrations; citric acid (50%, in side arm), 0.3 ml. Total volume, 2.0 ml. Aniline-citrate added afterwards, 0.3 ml.; Atmosphere, 10% CO_2 in N_2. Time, 50 minutes. Temperature, 30.4° C.

Thus it has been demonstrated that in the presence of optimal concentrations of pyruvate, carbon dioxide, and the enzyme, and in the absence of hydrogen gas or other suitable hydrogen donators, the presence of a small amount of oxalacetate or a compound very closely resembling it can be detected.

It has been previously demonstrated that there are at least two mechanisms for the formation of succinic acid with this enzyme preparation. Thus fumarate may arise from succinate which is formed by acetic acid condensation. However,

Table 31. Necessity for CO_2 and optimal concentration of $NaHCO_3$ for formation of oxalacetate.

Experiment No.	Conditions	Oxalacetate formed (µl.)		
			Average	Controls
1	NaOH	6		
		0		0
		9		0
		0	4	0
2	--	20		
		19	20	-2
3	$NaHCO_3$ (0.1 ml.)	21		
		30		0
		17	23	0
4	$NaHCO_3$ (0.2 ml.)	30		
		29	30	3
5	$NaHCO_3$ (0.3 ml.)	24		
		18	21	
6	$NaHCO_3$ (0.4 ml.)	24		
		23	24	2

NOTE: Each cup contained: juice, 0.8 ml.; pyruvate (0.3 M), 0.4 ml.; citric acid (1:1, in side arm), 0.3 ml. Cups in Experiment 1 contained, in addition, 0.3 ml. of 20% NaOH (in alkali well). Cups in Experiments 3-6 contained the indicated amount of 0.3 M $NaHCO_3$. Cups in Experiment 2 contained no NaOH or $NaHCO_3$. Atmosphere N_2. Time, 50 minutes. Temperature, 30.4° C. Total volume of cups made up to 2.0 ml. with water. Citrate-aniline added afterwards, 0.4 ml.

under anaerobic conditions, no oxalacetate was detected with fumarate as substrate. Another possibility is that oxalacetate may be formed from fumarate anaerobically, in the presence of a suitable hydrogen acceptor such as pyruvate. Under these conditions, however, fumarate is a much better hydrogen

acceptor than pyruvate, and thus the possibility of the anaerobic formation of oxalacetate from fumarate would seem to be eliminated. To completely eliminate the possibility, potassium cyanide was used as a selective inhibitor. At a concentration of 0.0025 M potassium cyanide dissimilation of pyruvate was inhibited 93%, according to the carbon dioxide evolution from bicarbonate buffer, and 95%, according to pyruvate actually dissimilated; but the formation of oxalacetate from pyruvate was not inhibited. These results correspond with those of Wood and Werkman (1940) in work with propionic acid bacteria, where it was found that potassium cyanide did not inhibit CO_2-fixation. Thus the dissimilation of pyruvic acid to acetic and formic acids was almost completely stopped with no decrease in the amount of oxalacetate formed.

Attempts to increase further the carboxylation of pyruvate have met with little success. However, it was found that in the presence of KCN after 2-3/4 hours the amount of oxalacetate formed was increased to 36 μl. (Table 32). Without cyanide present the amount was greatly decreased. The Straub colorimetric test was also used for the determination of oxalacetate and the amounts of oxalacetate formed, as determined by this method, checked well with the amounts formed as determined with the citrate-aniline method (Table 32).

In order to demonstrate conclusively the formation of oxalacetate from pyruvate and $C^{13}O_2$, the presence of an excess

Table 32. Comparison between the aniline-citrate and colorimetric methods in determination of oxalacetate from pyruvate and CO_2.

Method	KCN (M.)	Oxalacetate formed (μl.)
Aniline-citrate	0.00	16
	0.00	24
	0.0025	36
	0.0025	36
Colorimetric	0.005	31
	0.005	32
	0.005	37

NOTE: Each cup contained: juice, 0.8 ml.; pyruvate, 0.06 M; KCN in indicated concentrations. In the aniline-citrate method, 0.3 ml. citric acid (50%) was placed in side arm; 0.3 ml. aniline-citrate was added afterwards. In colorimetric method, 0.3 ml. of 10% trichloracetic acid was used as deproteinating agent. Total volume of cups, 2.0 ml. Atmosphere, 10% CO_2 in N_2. Time, 2.75 hours for aniline-citrate method, 2 hours for colorimetric method. Temperature, 30.4° C.

of heavy carbon would have to be demonstrated in the carboxyl group adjacent to the methylene carbon of the oxalacetate formed. This has not yet been accomplished because of the small amounts of oxalacetate present. Chemical analyses of the oxalacetate formed would also have to be made to determine whether the biological form is identical with the synthetic compound prepared in the laboratory. At any rate, the possibility still remains that phosphorylated or other intermediates are involved in the fixation reaction and that the

reaction is more complex than represented.

Summary and conclusions

The enzyme exhibits strong activity with fumarate and oxalacetate as acceptors of gaseous hydrogen. In addition, oxalacetate is rapidly decarboxylated. Manganese is necessary for the decarboxylation of oxalacetate, whereas cocarboxylase and inorganic phosphate are not.

There is no dilution effect of the enzyme in the decarboxylation of oxalacetate, although the specific protein nature of the enzyme concerned in this reaction was demonstrated.

The enzyme forms oxalacetate (or a closely related compound) from fumarate and malate, and smaller amounts from succinate aerobically. No oxalacetate is formed from fumarate anaerobically.

During decarboxylation of oxalacetate, in the presence of $NaHC^{13}O_3$, an exchange reaction was found to take place, with an excess of C^{13} located in the carboxyl group adjacent to the methylene carbon of the residual oxalacetate.

In attempts to demonstrate the carboxylation of pyruvate by the formation of oxalacetate from pyruvate and CO_2, no oxalacetate was detected under optimal conditions for CO_2-fixation by the juice under an atmosphere of 5% CO_2 in H_2.

Under the same conditions, but substituting nitrogen for hydrogen, definite tests were obtained for the formation of oxalacetate (or a compound very closely resembling it) from pyruvate and CO_2.

The effects of enzyme, pyruvate, and carbon dioxide concentration on the formation of "oxalacetate" are considered. Varying the concentration of enzyme and substrate resulted in the formation of different amounts of oxalacetate.

In the presence of 0.0025 M. potassium cyanide, the dissimilation of pyruvate was inhibited 93 to 95%, with no decrease in the amount of oxalacetate formed.

Under optimal conditions and in the presence of potassium cyanide, the quantity of oxalacetate formed was increased slightly.

Two different methods were used to determine the quantities of oxalacetate formed from pyruvate and CO_2. The results of both methods were in good agreement.

DISCUSSION

It has been demonstrated that pyruvic acid plus carbon dioxide under the correct conditions will combine to form oxalacetic acid or a compound very closely related to it. At this point, it might be interesting to pose some questions which naturally arise in connection with this reaction. For example, what role (either direct or indirect) does phosphorylation play in CO_2-utilization and what is the source of energy which enables heterotrophic assimilation of carbon dioxide to take place? No work has as yet been done on this phase of the problem.

Although carboxylation has been demonstrated, we are not certain whether pyruvate as such enters into the reaction or whether a phosphorylated form is the actual substrate. In order to demonstrate the reaction with phospho-pyruvate, the enzyme preparation used must not dephosphorylate the substrate. The next question which arises is, how can pyruvate be converted to phospho-pyruvate? This synthesis has not been demonstrated since Meyerhof et al. (1938) showed that the dephosphorylation of phospho-pyruvate to pyruvate is not a reversible reaction. Kidney extracts, which show a very rapid phosphorylation of different compounds aerobically (Kalckar, 1937), do not phosphorylate pyruvic acid. However, Kalckar did demonstrate the formation of phospho-pyruvate as a result of

oxidation of malate or fumarate by kidney extracts, and Lipmann (1941) proposed that the oxidation occurred by the addition of phosphoric acid to the double bond of fumaric acid:

$$\underset{\text{Fumaric acid}}{COOH.CH{:}CH.COOH} + H_3PO_4 \longrightarrow \underset{\text{Phosphofumaric acid}}{COOH.CH_2.CH.O.PO_3H_2.COOH}$$

$$COOH.CH_2.CH.O.PO_3H_2.COOH \xrightarrow{-2H} \underset{\text{Phosphooxalacetic acid}}{COOH.CH{:}C.O.PO_3H_2.COOH}$$

$$COOH.CH{:}C.O.PO_3H_2.COOH \xrightleftharpoons{-CO_2} \underset{\text{Phosphopyruvic acid}}{CH_2{:}C.O.PO_3H_2.COOH}$$

Thus, phosphopyruvate presumably could arise from pyruvate by way of the carboxylation reaction, but pyruvate and not phosphopyruvate would be the compound actually undergoing carboxylation. The evidence at present is too meager to indicate whether phosphate is a part of the molecule undergoing phosphorylation, but it is conceivable that phosphorylation may play a part in CO_2-fixation by supplying the energy needed for this assimilation.

Assimilations or syntheses are endergonic biological reactions and therefore need energy. In order to obtain this energy they are coupled with exergonic processes which yield energy. Since CO_2-fixation is an assimilative reaction, and phosphorylations and dephosphorylations, thermodynamically are important biological reactions, there is the possibility that the energy for this assimilation might come from the

splitting of some phosphate compound. According to Borsook (quoted by Evans et al., 1943), the decarboxylation of oxalacetate yields 5250 calories:

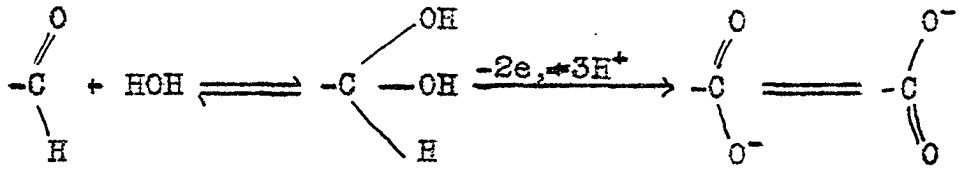
$$\underset{-184,210}{\text{oxalacetate}} + \underset{-56,200}{H_2O} \longrightarrow \underset{-106,460}{\text{pyruvate}} + \underset{-139,200}{HCO_3^-}$$

$$\Delta F = -5250$$

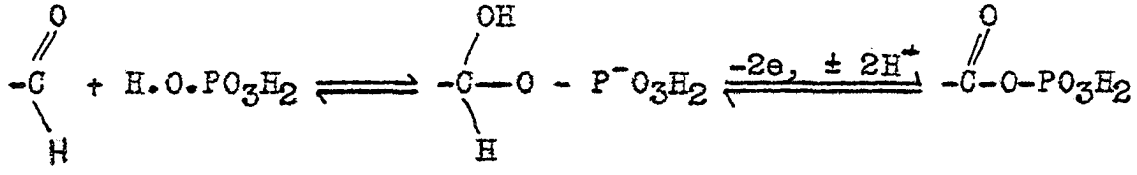
Therefore, the carboxylation of pyruvate would need that much energy. Examination of possible sources of such energy brings to light some possibilities closely connected with this reaction.

It is known that the formation and hydrolysis of pyrophosphate (for example, adenosine-triphosphate) are two of the most energy-rich reactions in biological systems. The free energy change (ΔF) involved in phosphorylation and dephosphorylation is not known, but the heat change (ΔH) has been measured. The ΔH in the splitting of pyrophosphate from adenosine-triphosphate amounts to about 11,000 calories per mol. P., and the ΔF (free energy change) has been estimated at more than 10,000 cal. per mol. P. (Kalckar, 1942). Thus, the dephosphorylation of adenosine-triphosphate theoretically could supply enough energy for the carboxylation of two molecules of pyruvic acid.

The energy in the pyrophosphate molecule can arise from the oxidation of carbonyl structures, coupled with phosphate uptake. Oxidation of an aldehyde group with water entering into the reaction



results in a large increase in stability of the carboxyl group which is formed, due to its resonating structure. This increase in stability is expressed in the liberation or scattering of a large amount of free energy. However, if instead of water phosphoric acid enters into the reaction,



there is little increase in the stability of the resulting compound because the carboxyl resonance and phosphate resonance are both eliminated and, therefore, only a very small change in free energy is involved. This simultaneous elimination of two resonating structures is referred to as opposing resonance. Therefore, instead of the energy being scattered and lost on oxidation, it is conserved. On transfer of phosphate groups, for example, to adenylic acid, adenosine-triphosphate is formed which contains most of the potential energy of the original carbonyl group. The hydrolysis of the pyrophosphate structure of adenosine-triphosphate results in the liberation of approximately 10,000 calories. Other structures in which opposing resonance occurs and which on dephosphorylation are converted to structures of high stability with the consequent

liberation of a large amount of free energy are carboxyl phosphate (such as 1,3-diphosphoglyceric acid, acetyl phosphate), guanidine phosphate (such as creatine phosphate) and pyrophosphate (such as adenosine-triphosphate).

Lipmann (1941) estimated the ΔF of the reaction phosphoenol-pyruvate $\longrightarrow$ pyruvate + phosphate to be about -11,250 cal. per mol. P. This reaction could be regarded as a possible source of energy for the carboxylation reaction. This might explain why pyruvate would be the compound actually undergoing carboxylation, since the dephosphorylation of phosphoenol-pyruvate might actually be necessary for carboxylation to take place.

The formation of acetyl phosphate may be another important step indirectly connected with carboxylation. Lipmann, by assuming the entry of phosphate into compounds with carbonyl groups, discovered acetyl phosphate. He established that the oxidation of pyruvate proceeded through an intermediate phosphate to acetyl phosphate. Acetyl phosphate is quite unstable and quickly breaks down at room temperature to acetic acid and inorganic phosphate. However, in the presence of adenylic acid adenosine-triphosphate is formed (Lipmann, 1941) which is ready to provide its high energy of dephosphorylation (approximately 10,000 cal.) to other chemical reactions. Confirmation of the occurrence of acetyl phosphate, this time under anaerobic conditions, during the dissimilation of pyruvate by an enzyme preparation of E. coli, has recently been

obtained (Utter and Werkman, 1943). It was also demonstrated that acetyl phosphate transfers phosphate to adenylic acid presumably forming adenosine-triphosphate.

Thus, during the anaerobic dissimilation of pyruvate, two reactions occur side by side: one, carboxylation, which requires energy; the other, the formation of acetyl phosphate and adenosine-triphosphate which, on splitting into adenylic acid and inorganic phosphate, yields energy. It is tempting to think that these two reactions may be complementary.

SUMMARY AND CONCLUSIONS

In attempts to modify and possibly improve the method used in obtaining active bacterial extracts, whereby the cells are ground with powdered Pyrex glass, it was found that more active preparations were obtained when less glass was mixed with the bacterial cells. Extensive grinding reduced the activity of the bacterial enzyme systems on glucose and hexosediphosphate. In a comparison of grinding agents, it was found that glass was superior to carborundum. The activity of the bacterial enxyme preparations was shown to be independent of the small amount of cells present.

By varying the media and conditions of growth, an enzyme preparation was obtained from Escherichia coli which was active in the anaerobic dissimilation of pyruvate, producing acetic, formic, lactic, and succinic acids and carbon dioxide. The preparation also contained the enzymes formic dehydrogenase and hydrogenase. The whole system could be frozen and dried in vacuo without any immediate loss in activity.

It was found by dialyzing the enzyme preparation that inorganic phosphate, manganese ions and cocarboxylase were components of the enzyme system in the anaerobic dissimilation of pyruvate.

Under the correct conditions an actual net uptake of carbon dioxide by this bacterial enzyme preparation was

demonstrated with pyruvate as substrate. In the presence of carbon dioxide containing an excess of heavy carbon (C^{13}) the fixed carbon dioxide was traced to the carboxyl groups of the succinic, lactic, and formic acids formed. Again, making use of the tracer technique, another mechanism for succinic acid formation besides that of CO_2-utilization was demonstrated, i.e., the condensation of acetic acid, or its derivative, to form succinic acid. The enzyme preparation did not contain hydrogenlyase, and in the absence of this enzyme it was demonstrated that formic acid does not arise by a reduction of carbon dioxide in the medium but more probably is split from pyruvate.

The enzyme preparation exhibited strong activity on oxalacetate and fumarate, postulated intermediates in the fixation reaction. Quantitative data are presented on the decarboxylation of oxalacetate, the reduction of oxalacetate and fumarate, and the reverse formation of oxalacetate from succinate, fumarate, and malate. During the decarboxylation of oxalacetate in the presence of $C^{13}O_2$, an exchange reaction takes place between the carboxyl group adjacent to the methylene carbon atom of oxalacetate and the CO_2 in the medium. Finally, with pyruvate and CO_2 as substrate and in the presence of the enzyme preparation, small amounts of oxalacetic acid or a compound very similar to it were detected. The quantities of "oxalacetic" acid formed depend on optimal concentrations of the enzyme and pyruvate, and it was shown that carbon dioxide

was necessary for the formation of this compound.

The evidence presented points to the accuracy of the original mechanism postulated for the Wood-Werkman reaction. The application to intact cells of results obtained with cell-free enzyme preparations does not necessarily completely follow. However, keeping in mind what the living cell is and the complexity of the enzyme systems, cell-free preparations can be advantageously employed in attempts to elucidate some of these complex biochemical activities.

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